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Corsa et al.

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(54) **PROCESS FOR THE PRODUCTION OF HYALURONIC ACID IN *ESCHERICHIA COLI* OR BACILLUS MEGATERIUM**

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C12P 19/04	(2006.01)
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C12N 9/12	(2006.01)
C12N 9/92	(2006.01)
C12N 15/75	(2006.01)

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(58) **Field of Classification Search**

CPC C12Y 101/01022; C12Y 204/01212; C12Y 207/07009; C12Y 503/010009

See application file for complete search history.

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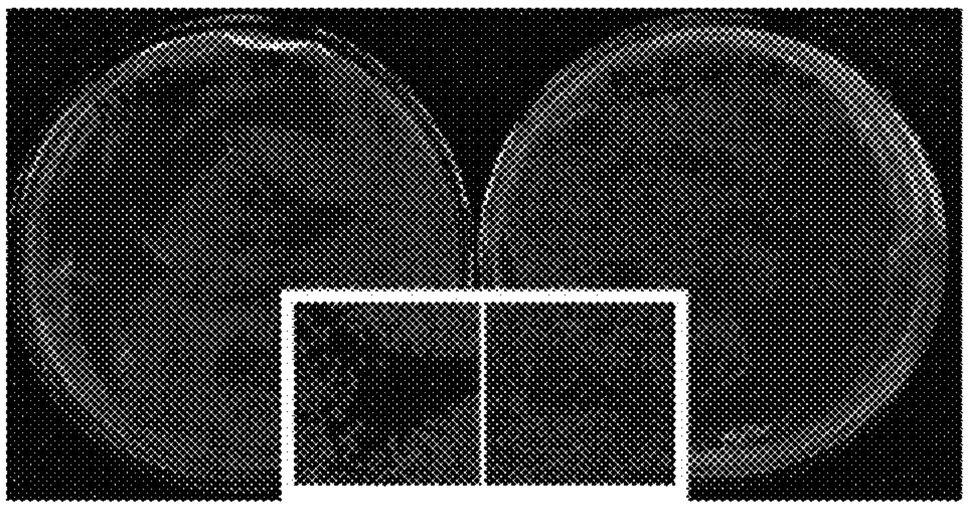
(57) **ABSTRACT**

A method of producing hyaluronic acid (HA) in *Escherichia coli* and *Bacillus megaterium* through episomal plasmid vectors wherein the gene is under the control of strong promoter T7, preferably under the control of strong promoter T7 of bacteriophage T7, and a system for the selection of stable bacterial strains producing high levels of hyaluronic acid, are provided.

32 Claims, 6 Drawing Sheets

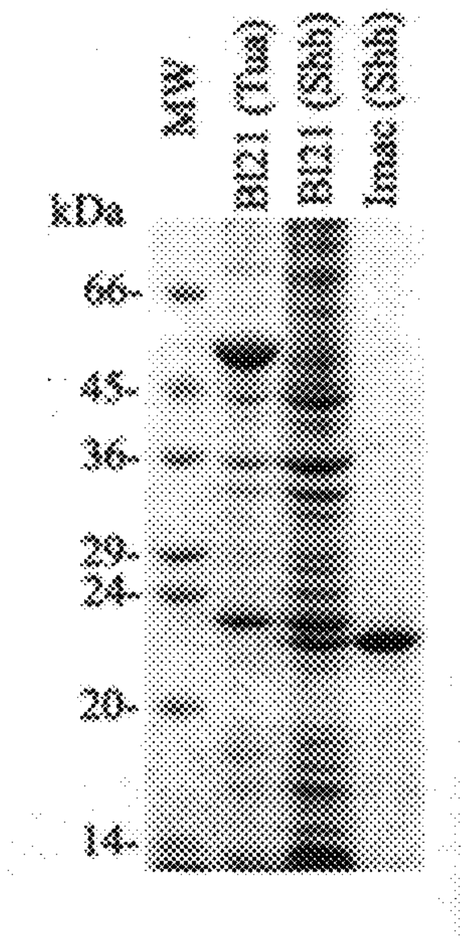
HAS1 e TUAD expression in E.coli TOP-10



Cells incorporating plasmide HT01 are larger than cells with BS3 (difficulty to grow)

(Cells incorporating BS3 are more yellow than parental cells)

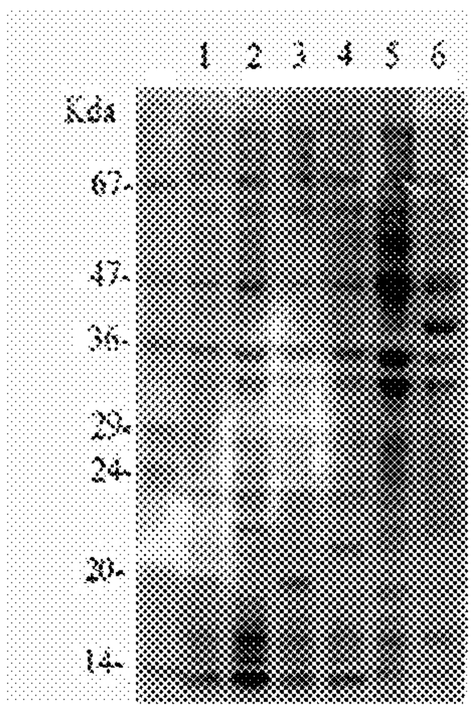
Fig. 1



TuaD expression in E coli BL21 DE3

Fig. 2

Constitutive expression of Hyaluronan synthase (Streptc) in E. coli



equisimilis. The encoded protein, designated seHAS, is 313 amino acids long calculated molecular weight, 37,778; calculated pI, 9.3) and is the smallest member of the HAS family identified thus far. The enzyme migrates anomalously fast in SDS-polyacrylamide gel electrophoresis (~42,000 Da). The seHAS protein shows no sim-

Fig. 3

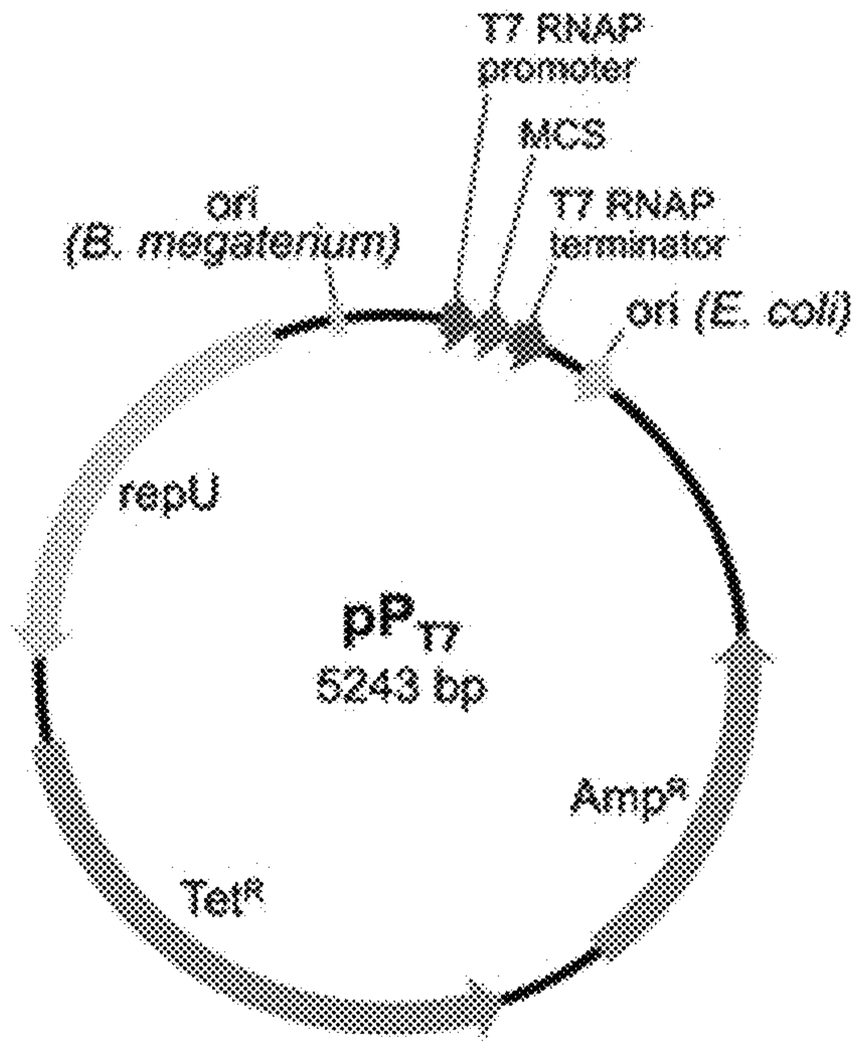


Fig. 4

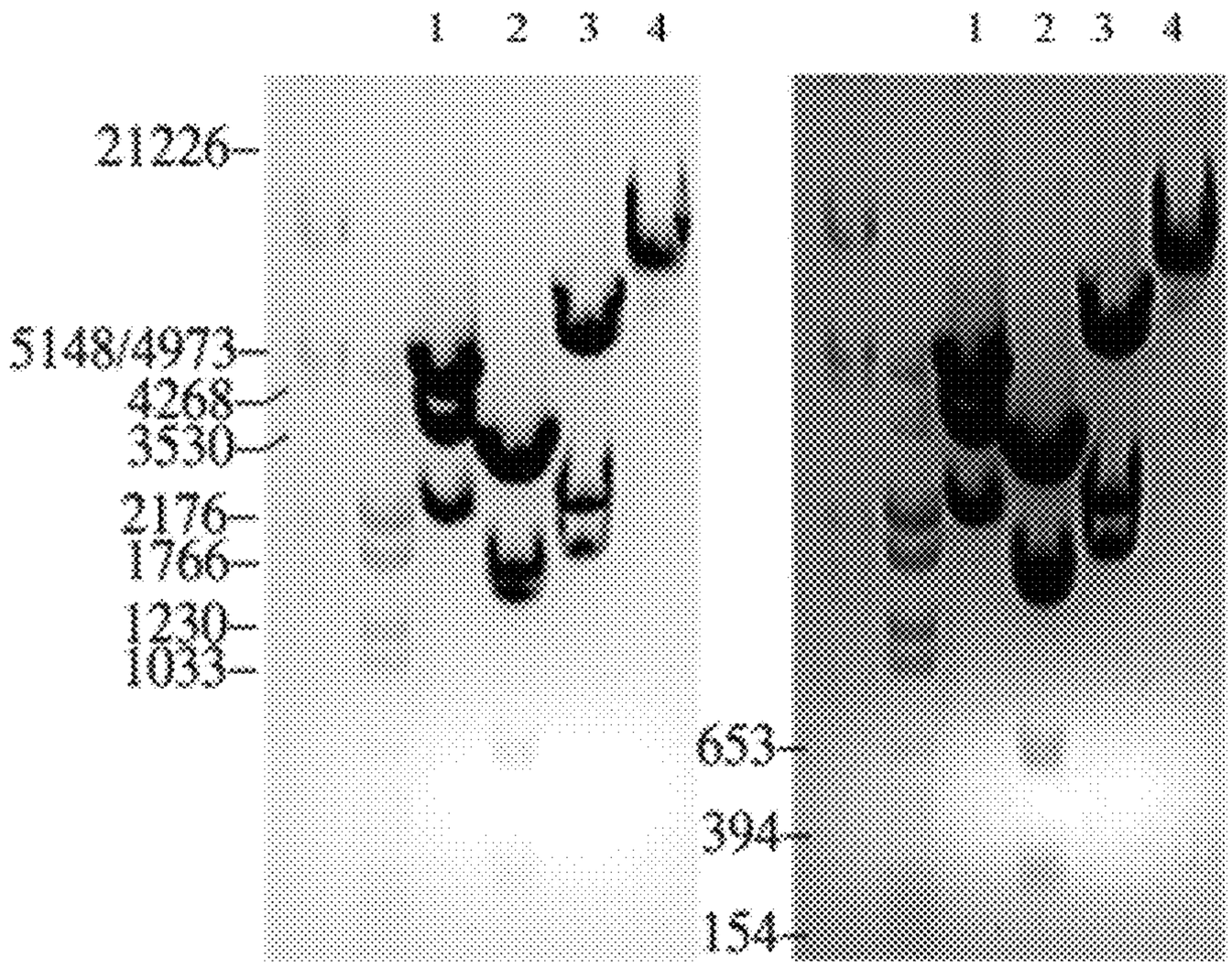

1 2 3 4
21226
5148/4973
4268
3530
2176
1766
1230
1033
1 2 3 4
653
394
154

Fig. 5

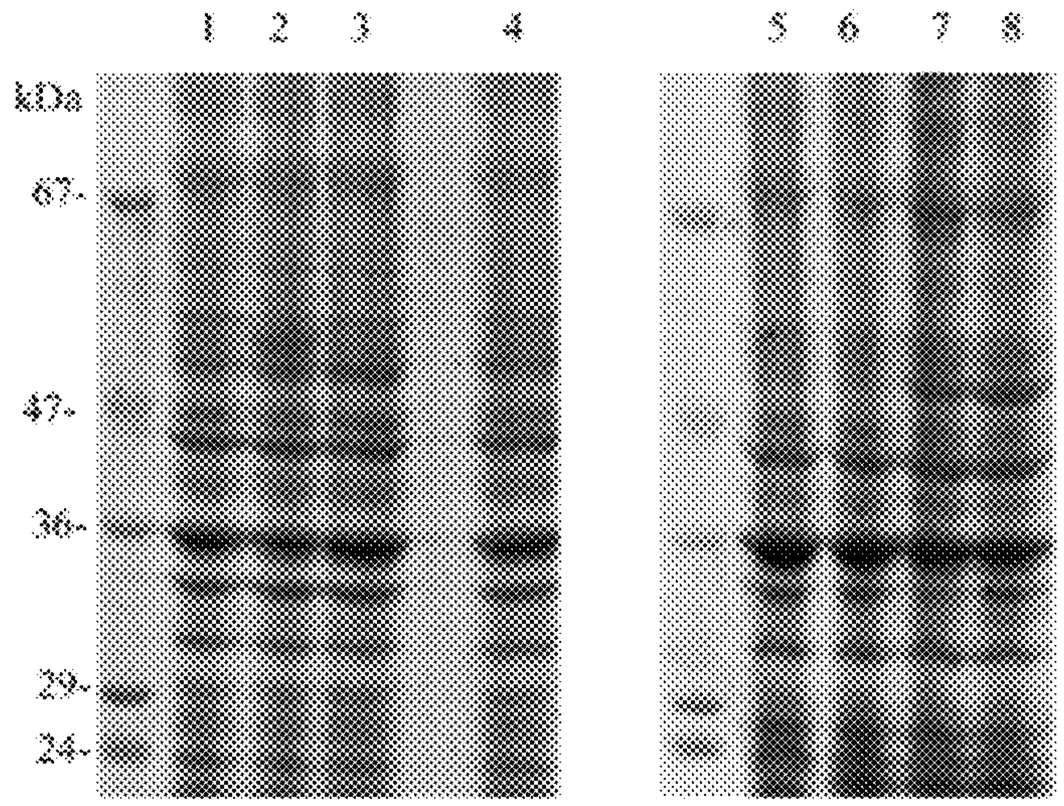

1 2 3 4
5 6 7 8
kDa
67
47
36
29
24

Fig. 6

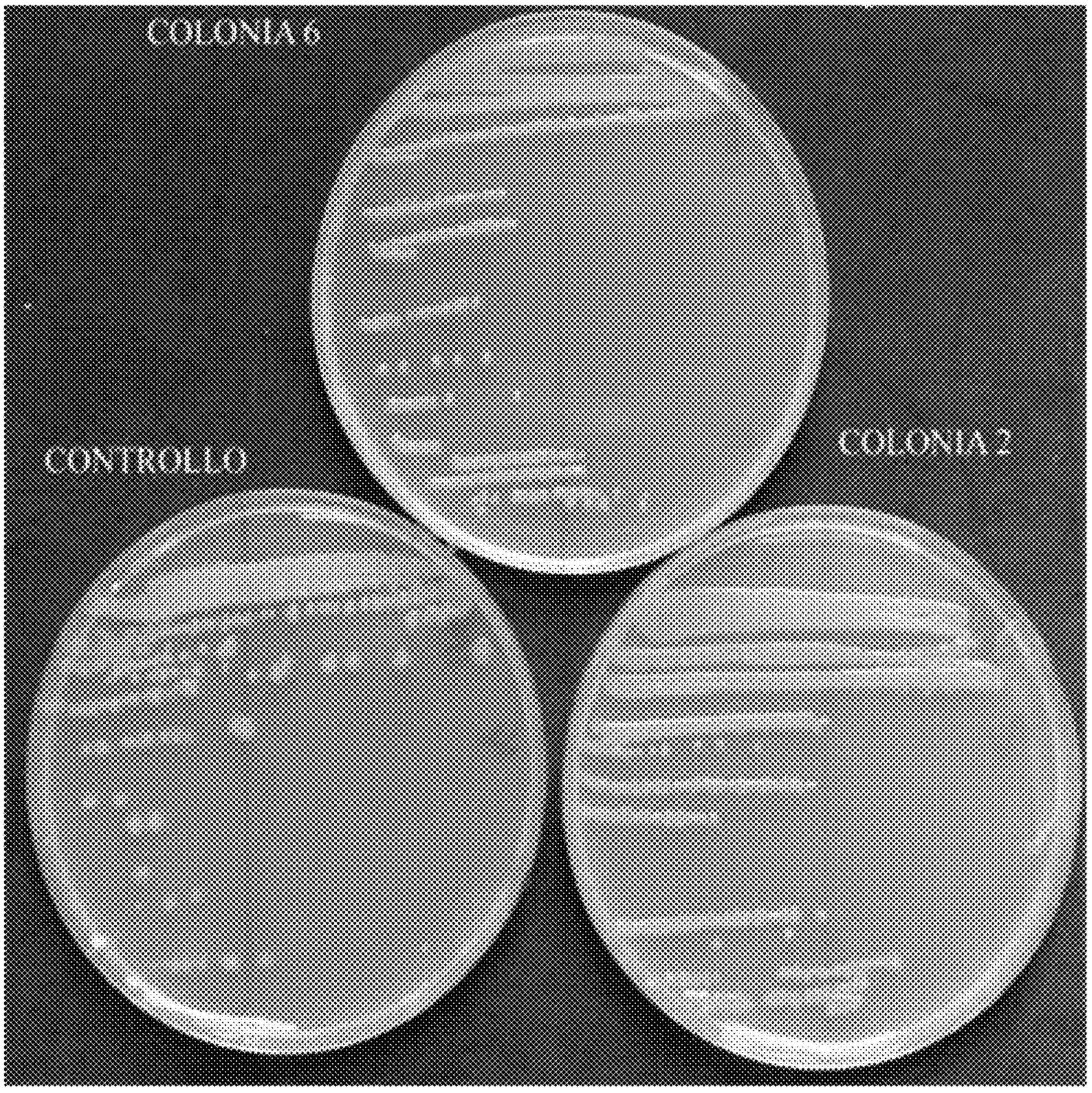


Fig. 7

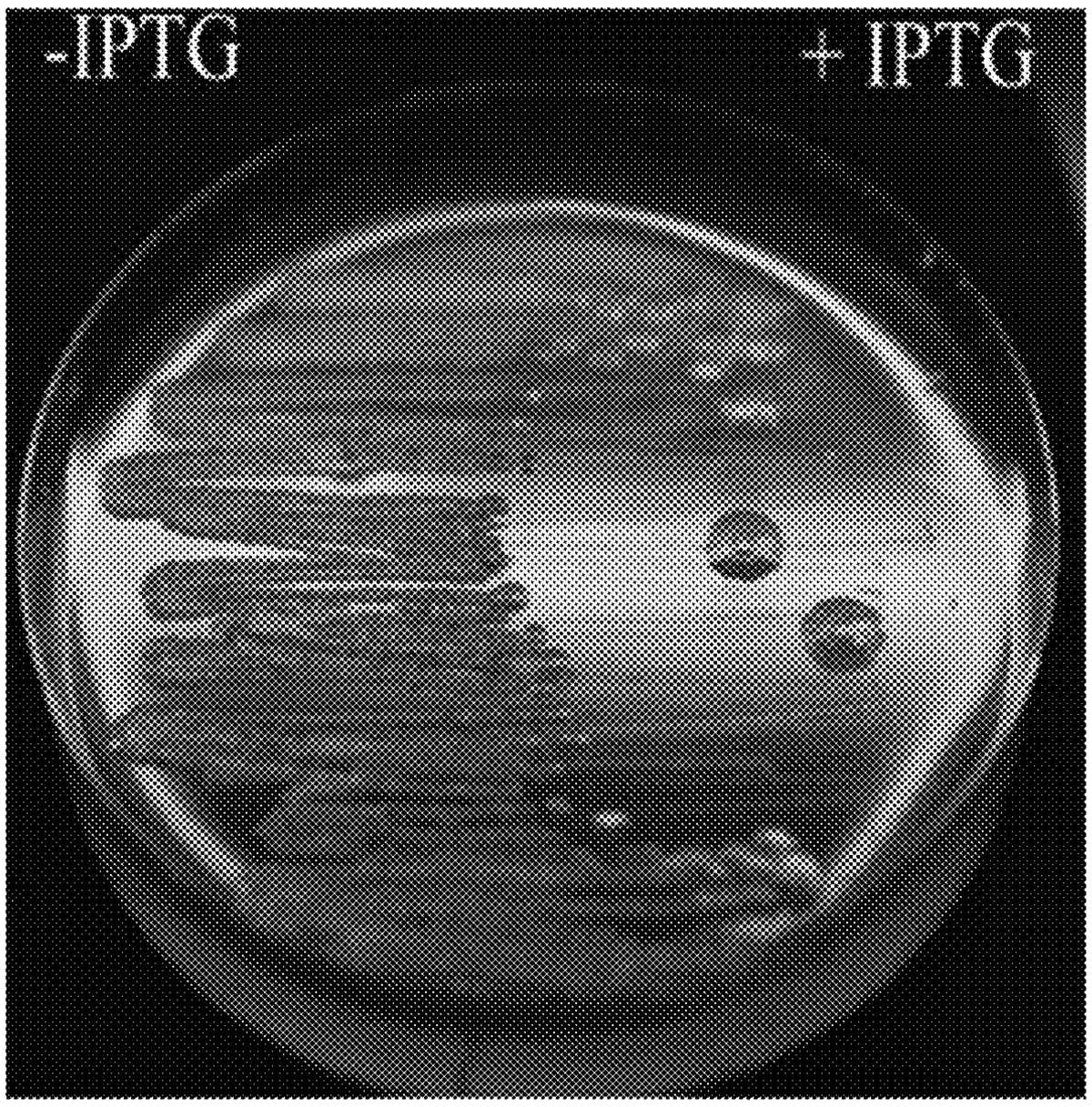


Fig. 8

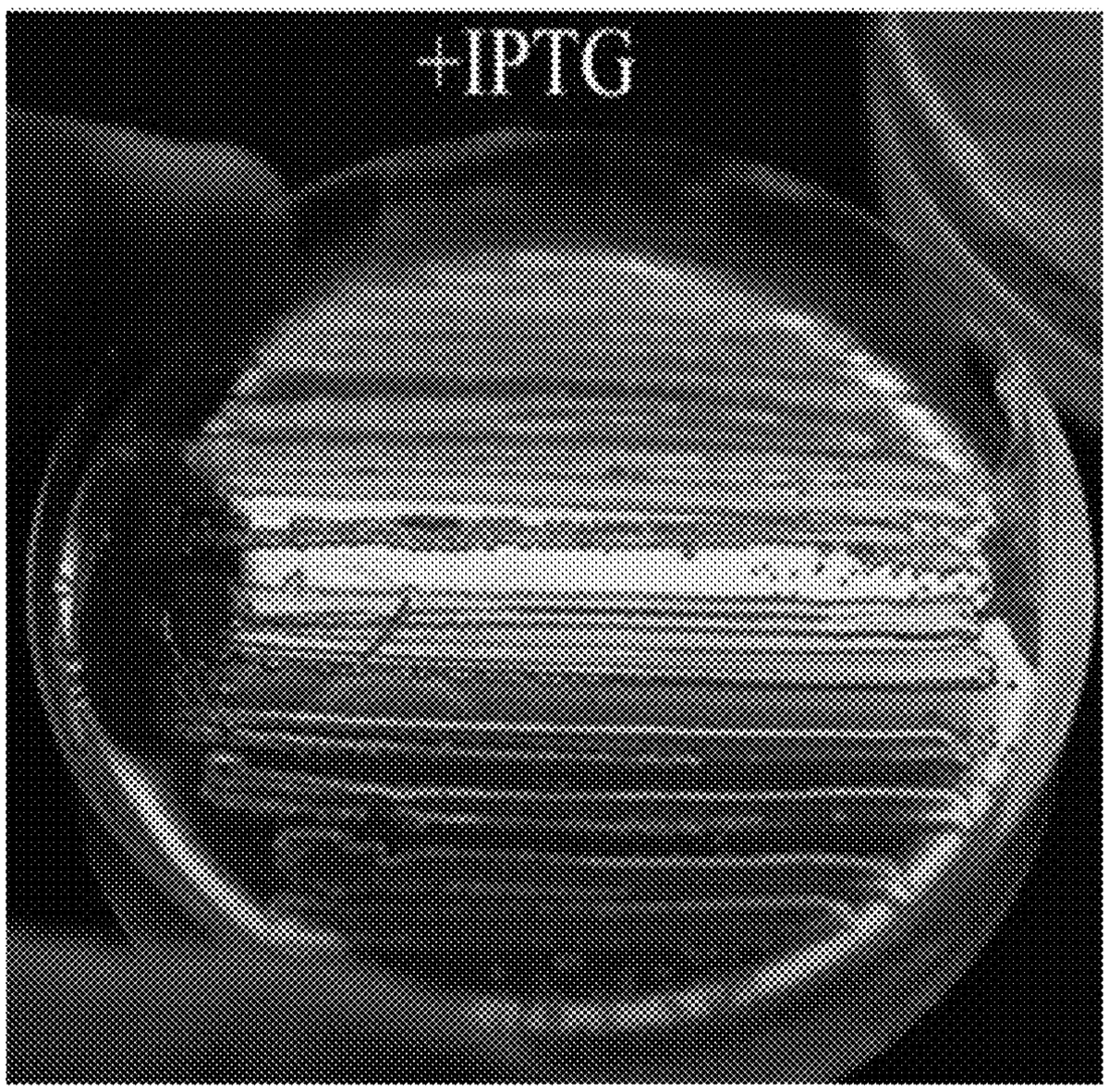


Fig. 9

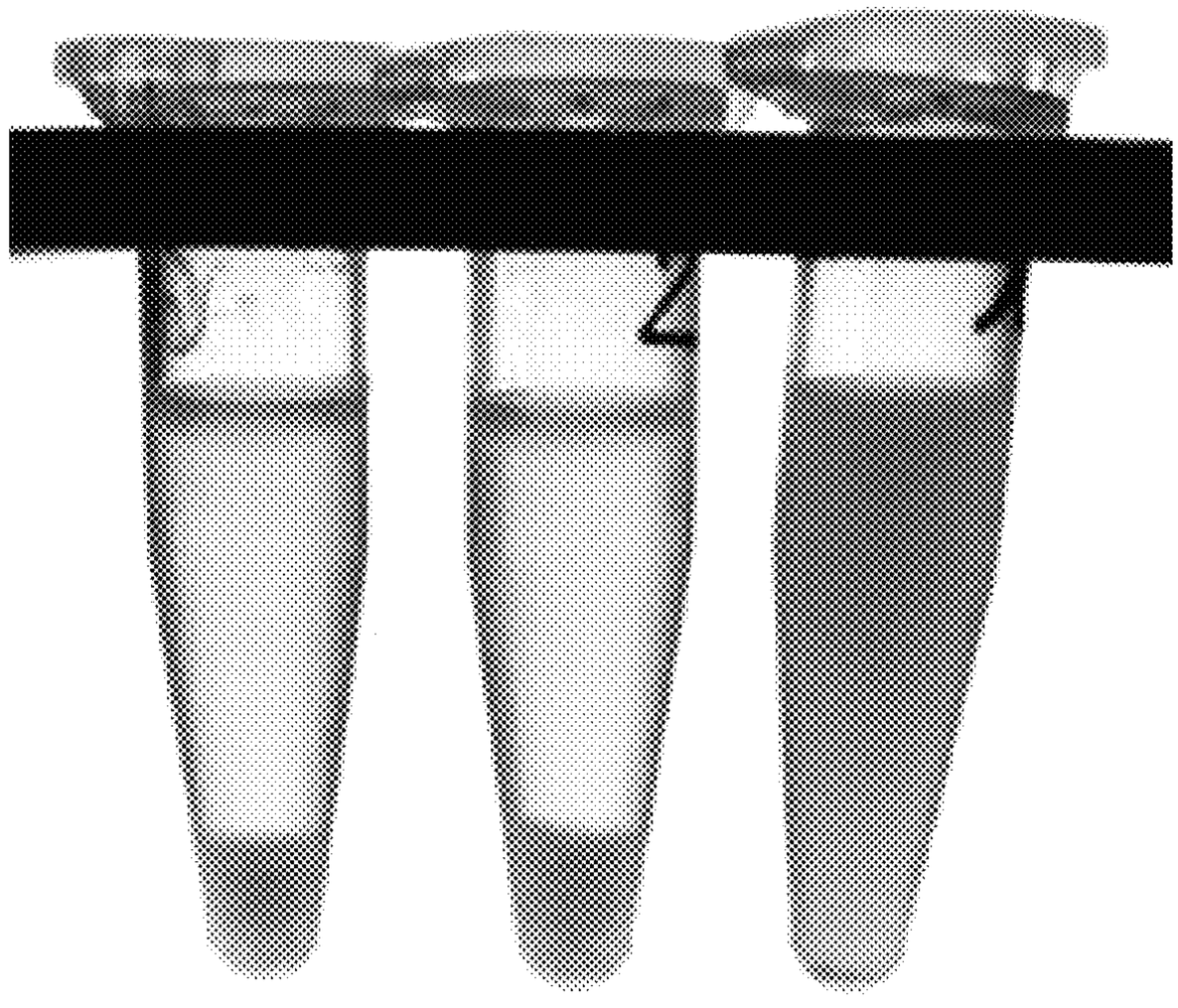


Fig. 10

PROCESS FOR THE PRODUCTION OF HYALURONIC ACID IN *ESCHERICHIA COLI* OR BACILLUS MEGATERIUM

This application is a Continuation of copending application Ser. No. 13/821,953, filed on Apr. 15, 2013, which was filed as the National Phase of PCT International Application No. PCT/EP2011/065641 on Sep. 9, 2011, which claims the benefit under 35 U.S.C. §119(a) to Patent Application No. MI2010A001641, filed in Italy on Sep. 9, 2010, all of which are hereby expressly incorporated by reference into the present application.

SUBJECT OF THE INVENTION

The present invention discloses a process for the production of hyaluronic acid (HA) in *Escherichia coli* and *Bacillus megaterium* through episomal plasmid vectors wherein the gene is under the control of a strong T7 promoter, preferably under the control of a strong T7 promoter of bacteriophage T7, and a system for the selection of stable bacterial strains producing high levels of hyaluronic acid.

FIELD OF INVENTION

Hyaluronic acid is a natural linear polysaccharide which consists of alternating β-1-4 D-glucuronic acid and β-1-3 N-acetyl glucosamine. Hyaluronic acid is part of the glycosaminoglycan family, and can reach a molecular weight of 10^7 Da, with approx. 300000 repeating saccharide units. It is widely distributed in the connective tissue and extracellular matrix in the epithelium of eukaryotic organisms, where it is located on the cell surface, but can also be synthesised in some prokaryotic organisms, such as those of the *Streptococcus* family. Glycosaminoglycans are ideal joint lubricants, but also perform many other functional roles in tissue repair, cell motility, adhesion and development, cancer and angiogenesis. Products based on hyaluronic acid have been developed on the basis of these important characteristics, and are used in orthopaedics, rheumatology and dermatology.

The most common natural sources of HA include rooster combs, the classic material from which HA is extracted, and some bacteria, especially those belonging to the *Streptococcus* family. All these different sources present numerous disadvantages: hyaluronic acid obtained from rooster combs can, for example, cause allergies in humans because it is of avian origin, while HA from bacterial sources must be free of all the toxins normally present in those bacteria which can cause possibly serious immune/inflammatory reactions. The current industrial HA purification processes therefore comprise many different steps, with a consequent increase in the final costs of manufacturing the raw material.

There is consequently a strongly felt need for alternative sources that eliminate all the adverse events described, while maintaining reasonable manufacturing costs. In recent years, biosynthesis pathways for the synthesis of hyaluronic acid have been included in detail in numerous organisms. While the genes required for hyaluronic acid synthesis which are present in eukaryotic organisms are distributed throughout the genome, in bacterial systems said genes are often present and organised in operons. For example, in *Streptococcus equi* the operon for hyaluronic acid comprises 5 genes: hasA, hasB, hasC, hasD and hasE. Sometimes, however, the genes are present in two operons: in *Streptococcus equisimilis* one operon with genes hasA, hasB and hasC is present, and another with genes hasC, hasD and hasE. The genes

homologous with hasB, hasC, hasD and hasE of the Streptococci are present in many organisms, and synthesise the enzymes necessary for the synthesis of hyaluronic acid precursors D-glucuronic acid and N acetyl-D glucosamine, which are also the essential constituents of the bacterial walls. In the case of streptococci, hyaluronan synthase (hasA, which is present in the plasma membrane) is the key enzyme for the final synthesis of hyaluronic acid because it performs two functions: it catalyses the union of D-glucuronic acid and N-acetyl-D-glucosamine, and transports the chain of newly-formed hyaluronic acid out of the cell. The study of the enzymes responsible for hyaluronic acid synthesis has allowed the development of recombinant systems in various organisms, such as *Bacillus subtilis, Lactococcus lactis, Escherichia coli* and *Agrobacterium radiobacter*. The first organism engineered to produce hyaluronic acid was *B. subtilis*, through cloning in its chromosome of an operon that carries the hasA gene from *Streptococcus* (which is missing in *Bacillus*), with the tuaD and gtaB genes of *Bacillus* (corresponding to hasB and hasC of *Streptococcus*), under the control of a constitutive promoter (US2003/175902). In this way a biosynthesis pathway was organised in operons similar to those of *Streptococcus equi*, one of the major natural producers of hyaluronic acid. However, the system thus perfected leads to the industrial production of a hyaluronic acid with a weight average molecular weight of less than 1 MDA, with very low manufacturing yields.

The system of expression of hyaluronic acid according to the present invention uses bacteria of the strains *Bacillus Megaterium* and *Escherichia coli.*

Bacillus Megaterium is an aerobic gram-positive bacterium, which was described over 100 years ago. Its large size (1 μm, i.e. 100 times larger than *E. coli* in both vegetative and spore-forming form) has made it very popular for morphological analysis studies. This bacterium can contain many different types of plasmids; the plasmid DNA can be transferred by protoplast transformation obtained by treatment with polyethylene glycol, and they all work extremely well, with excellent structural stability. The bacterium can be transduced with phages, and the frequency of transformation can reach 10^6 transformants per μg of DNA. Several hundred mutants are currently available, which cover various biosynthesis pathways: catabolism, division, sporulation, germination, antibiotic resistance and recombination.

No less than seven plasmids have been found in different strains of *B. megaterium*, with sizes ranging from 5.4 to 165 kb. The genomes of two strains (DSM319:EMBL, accession number CP001983, and QM B1551:EMBL, accession number CP001982) and those of the seven natural plasmids are now available. Although it is considered to be a bacterium present in soil, *B. megaterium* has been found in various ecological niches such as dried meat, seawater and fish. *B. megaterium* is able to grow in various carbon sources, including slaughter waste and industrial syrups with a broad spectrum of sugars (62 of the 95 tested), which include carboxylic acids like acetate. *B. megaterium* can be cultured at high density, up to 80 g of dry weight per litre. Considerable knowledge has been obtained of various recombinant enzymes with different industrial applications which can be secreted in this organism, such as α-amylase, β-amylase, penicillin amidase, neutral protease and β-glucanase. Particularly important are amylases, used in the bread-making industry, glucose dehydrogenase, used industrially for the production of NADH and as a biosensor, and penicillin

amidase, used to generate new synthetic antibiotics. Finally, *B. megaterium* is the major source of vitamin B12.

DETAILED DESCRIPTION OF THE INVENTION

The present invention discloses and claims a process for the production of hyaluronic acid (HA) in high industrial yields in *Bacillus megaterium* and *Escherichia coli* through episomal plasmid vectors wherein the genes for the synthesis of the enzymes required for HA production, are under the control of the strong T7 promoter, preferably under the control of the strong T7 promoter of bacteriophage T7, and a system for the selection of engineered, stable bacterial strains producing high amounts of hyaluronic acid having well defined weight average molecular weights (in the following also indicated as MW).

In order to produce recombinant proteins (in this case the enzymatic proteins required for the synthesis of HA) efficiently, systems which use highly controllable strong promoters need to be designed. The invention discloses a process for the transformation of the above-disclosed bacteria, using a very efficient system for the control of the transcription of the genes introduced, as the gene of interest is placed under the control of the promoter dependent on T7 RNA polymerase.

During construction in *E. coli* of the vectors expressing hyaluronic acid in the form of plasmids, it was discovered that the genes thus introduced (which are responsible for synthesis of the hyaluronic acid-producing enzymes) are cell-toxic when their transduction control is a strong constitutive promoter. In fact, in *E. coli* transformed with genes hasA and tuaD, gene transduction of hasA alone leads to a great reduction in the D-glucuronic acid precursors required to constitute the bacterial wall, with the result that the cell dies, whereas gene transduction of tuaD alone generates uncontrolled synthesis of D-glucuronic acid which, by acidifying the bacterium and depriving it of glucose (its precursor), causes its death. Conversely, the transduction of both genes by bacterial polymerases leads to the activation of the two enzymes at different times, because they require different construction times with different procedures and sites of action (for example, hasA is a transmembrane protein with different domains crossing it, so a much longer time is needed for its synthesis and correct folding). The cell can only survive if balanced quantities of the precursor enzymes and the enzyme necessary for hyaluronic acid synthesis are present. In this case, the excess D-glucuronic acid, which is toxic at high levels in the cell, is used by hyaluronan synthase (hasA) which, combining it with glucosamine, incorporates it in the nascent hyaluronic acid and exports it from the cell, thus keeping the cell alive.

Consequently, although both hasA and tuaD are necessary for the synthesis of hyaluronic acid, it is essential for the two genes to work in concert, leaving the cell the time required to:

- produce D-glucuronic acid at non-toxic levels and
- trigger the transcription of the hasA gene in such a way that the latter is able to dispose of the high levels of D-glucuronic acid as they accumulate in the cell.

In the present invention, the problems described above have been solved by

- placing the plasmid genes, necessary for the synthesis of the above disclosed enzymes, under the control of a T7 promoter, preferably under the control of the T7 promoter of bacteriophage T7, dependent on T7 RNA polymerase, which uses repressor Xy1R in *B. megaterium* (and lac in *E. coli*) for its induction. The T7 promoter of bacteriophage T7 is dependent on the presence of T7 polymerase, so the HA synthesis genes placed under its control can only be transcribed by T7 RNA polymerase, not by the action of the polymerases naturally present in the bacterium;
- perfecting a system of selection of stable, engineered and secreting *B. megaterium* strains and E. *Coli* strains, preferably of viable, engineered and secreting *B. megaterium* strains, wherein the enzymes necessary for the HA synthesis, are present in "balanced" amounts, thus non toxic for the cell.

It is therefore object of the present invention a process for the preparation of hyaluronic acid in *Escherichia coli* or *Bacillus megaterium*, preferably in *B. megaterium*, comprising the following steps:

(a) culture of bacterial host cells of *Escherichia coli* or *Bacillus megaterium*, preferably of host cells of *B. megaterium*, transformed in a stable way with the T7 RNA polymerase system under conditions suitable for the production of hyaluronic acid in the presence of isopropyl-β-thiogalactopyranoside (IPTG) or xylose respectively as inductors, wherein said bacterial host cells are characterised by being further transformed with:

(i) at least one episomal plasmid vector comprising a sequence coding for the enzyme hyaluronan synthase and a sequence coding for the enzyme UDP-glucose dehydrogenase in tandem under the control of the strong inducible T7 promoter, preferably under the control of the T7 promoter of bacteriophage T7; or

(ii) at least one episomal plasmid vector comprising a sequence coding for the enzyme hyaluronate synthase, a sequence coding for the enzyme UDP-glucose dehydrogenase, a sequence coding for the enzyme UDP-glucose pyrophosphorylase and a sequence coding for the enzyme glucose 6 phosphate isomerase, under the control of the strong inducible T7, preferably under the control of the T7 promoter of bacteriophage T7;

(b) recovery of hyaluronic acid from the culture medium, wherein such bacterial host cells of *Escherichia coli* or *Bacillus megaterium* transformed in a stable way with the T7 RNA polymerase system and with plasmid vector (i) or (ii) able to produce hyaluronic acid of step a) are pre-selected in the plate on IPTG or xylose gradient respectively.

The Applicant preferably used *B. megaterium* (preferably pertaining to QMB1551 or DSM319 strains), transformed with the T7 RNA polymerase system, for its subsequent transformation with the episomal plasmid containing the genes for HA synthesis, as it presents various advantages as host for the expression of heterologous DNA:

- the HA produced is easily secreted;
- it is free of exotoxins and endotoxins, unlike gram-negative bacteria;
- it does not contain any alkaline protease, and consequently does not induce the breakdown of the protein produced;
- it is structurally very stable by comparison with recombinant plasmids: *B. megaterium* can contain a much larger number of episomal plasmids than *Bacillus Subtilis*, which are more stable; *B. megaterium* can also support much larger inserts than *E. coli* and *B. subtilis*, and this characteristic is very important when, as in the case of the present invention, a long metabolic pathway like that of hyaluronic acid is to be engineered.

The T7 RNA polymerase system transferred to *B. megaterium* (and to *E. coli*, preferably to *E. coli* BL21 DE3 strain)

controls the expression of the genes responsible for synthesis of the HA biosynthesis pathway (cloned in episomal plasmids), and guarantees

very high activity and selectivity of gene transcription;

a consequent very high production of the recombinant proteins required for the synthesis of hyaluronic acid.

The final yield of the desired product will be very high: much higher than that obtained with *B. subtilis*, where the operon system is cloned on the chromosome of the bacterium, and is under the control of non-inducible constitutive promoters.

In fact, the T7 RNA polymerase system described above is inducible: it is introduced artificially into the bacterium and activated by the Applicant by adding substances like IPTG (for *E. coli* in quantities of between 0.1 mM and 10 mM, preferably between 0.4 and 1 mM) or xylose (for B. *Megaterium* in quantities of between 0.1% and 10%, preferably between 0.5% and 1% w/v); in their presence, the inducer bonds to the repressor, modifying its configuration, and the repressor then detaches from the promoter, allowing the polymerases of the bacterium to transduce the gene for synthesis of T7 RNA polymerase. The latter, in turn, can only activate the gene transcription of the genes placed under the control of a T7 promoter. In this way the synthesis of the whole biosynthesis process for the production of HA can be controlled. The system is so efficient in that a single polymerase is dedicated to the gene of interest, and the RNA polymerase of the bacterium is not involved. With this methodology, the cell protein synthesis system is saturated, so that the proteins of interest are obtained in amounts to 50% or more of the total proteins.

Further, as demonstrated in the following by the Applicant, by modulating the fermentation times, the Applicant can obtain the production of high amounts of HA with specific weight average molecular weights, comprised in a range of from 100 KD to above 2 MD. More particularly, when the process according to the invention uses bacterial host cells of *B. megaterium* and fermentation time is comprised of from 80 to 160 hours, it is possible to obtain HA having a weight average MW comprised in the range 100-500 KD; when fermentation time is comprised of from 40 to 80 hours, it is possible to obtain HA having a weight average MW comprised in the range 500-1000 KD; when fermentation time is comprised of from 12 to 40 hours, it is possible to obtain HA having a weight average MW comprised in the range $1\times106-3\times10^6$ D.

In a preferred embodiment of the present invention, the sequence coding for the enzyme hyaluronan synthase (hasA) is obtained from a *Streptococcus* strain, preferably from *Streptococcus zooepidemicus*, and the sequences coding for enzymes UDP-glucose dehydrogenase (hasB or tuaD), UDP-glucose pyrophosphorylase (hasC or gtaB) and glucose 6 phosphate isomerase (hasE or pgi), are derived from *B. subtilis*.

According to a particularly preferred embodiment of the present invention, the sequences coding for enzymes hyaluronan synthase, UDP-glucose dehydrogenase, UDP-glucose pyrophosphorylase and glucose 6 phosphate isomerase include an upstream Shine-Dalgarno sequence.

Even more preferably, said plasmid vector (i) comprises or consists of the nucleotide sequence as defined in SEQ ID NO:1 or in SEQ ID NO:2.

The subsequent purification of the HA secreted will be extremely simple, with the result that the industrial production process will be much cheaper than the process according to the state of the art.

Specifically, *E. coli* strains BL21 DE3 (Stratagene, Calif., USA) have T7 RNA polymerase cloned in the chromosome of the bacterium under the control of the inducible promoter lac. It can be induced with IPTG for the transcription of the T7 RNA polymerase gene. At this point, the T7 RNA polymerase produced can transcribe the genes under its control.

A similar system has also been engineered in *B. megaterium*. In this case the system uses two plasmids: the first leads to the synthesis of the enzymatic protein T7 RNA polymerase, and the second (engineered) to that of the messenger of the gene (or genes) of interest, under the control of the T7 promoter of bacteriophage T7. The first plasmid, pT7-RNAP (MoBiTec), derives from plasmid pBM100 264 (MoBiTec), which replicates in *B. megaterium* QM B1551 (MoBiTec) and also contains the replication origin of *E. coli*, resistance to ampicillin and chloramphenicol and the promoter for xylose PXy1A, and its repressor Xy1R, which control the synthesis of T7 RNA polymerase, whose gene sequence is in the same plasmid. The plasmid for synthesis of recombinant proteins, pPT7 (MoBiTec), derives from *B. cereus* and leads to a replication origin of *B. megaterium* and resistance to ampicillin and chloramphenicol, and a replication origin for *E. coli* and the promoter T7 controlled by T7 RNA polymerase.

When the protein of interest is to be synthesised, xylose is added to the cells, and activates its promoter by detaching the repressor. The promoter, freed, then allows the polymerase of the bacterium to transcribe the gene for synthesis of the T7 RNA polymerase enzyme which, moving onto the T7 promoter of the other plasmid, transcribes its gene of interest, namely the genes required for HA synthesis. The system is highly efficient, because a single polymerase is dedicated to the transcription of the gene of interest, and the multiple copies of the two plasmids ensure that the transcript levels are extremely high.

A further object of the present invention are plasmid vectors, containing the two genes hasA and tuaD or the four genes hasA, tuaD, gtaB and pgi (corresponding to hasE), under the control of T7 promoter of RNA polymerase of bacteriophage T7, which are suitable to allow the production in *B. megaterium* and/or in *E. coli*, preferably in *B. megaterium*, of hyaluronic acid in high yield, according to the methodology described above. Preferably, the sequences coding for the hyaluronan synthase enzyme, UDP-glucose dehydrogenase, UDP-glucose pyrophosphorylase and glucose 6 phosphate isomerase include an upstream Shine-Dalgarno sequence. These vectors can also be constructed so as contain any other gene relating to the biosynthesis of hyaluronic acid.

Unlike those available to date, the starting plasmid is small, which allows engineering of the entire hyaluronic acid biosynthesis pathway (i.e. the four genes hasA, tuaD, gtaB and pgi) in a single plasmid, which is herein referred to as pPT7hasAtuaDgtaBpgi, making the present invention economically advantageous and successfully applicable on an industrial scale. In a preferred embodiment of the present invention the plasmid vector is pPT7hasAtuaD (SEQ ID NO:1) or pPT7hasAtuaDgtaBpgi (SEQ ID NO:2).

The present invention also relates to a method and relative system for the production/construction of bacterial strains, transformed with plasmid containing the entire hyaluronic acid biosynthesis pathway, with the 2 genes or 4 genes, and the selection of stable, viable, replicating and HA-secreting bacterial strains with high yield.

Said method of construction of engineered strain with the 2 genes or 4 genes plasmid vector for the HA synthesis comprises the following steps:

- Cloning of the tuaD gene (UDP-glucose dehydrogenase) from *Bacillus Subtilis,*
- Cloning of the hasA gene (hyaluronan synthase) from *Streptococcus zooepidemicus,*
- Construction of the plasmid pGEM4hasA,
- Construction of a plasmid with the tuaD gene following hasA,
- Cloning of the hasA-tuaD gene in the plasmid for *B. megaterium* pPT7: pPT7hasAtuaD;

the process for the construction of the 4 genes route proceeds with the following steps:

- Cloning of the gtaB gene: construction of the plasmid pGEM4hasA-gtaB,
- Cloning of the pgi gene from *Bacillus Subtilis,*
- Construction of plasmid pPT7hasAtuaDgtaBpgi, which is referred to as pT7hyal,
- Transformation of plasmids pPT7hasAtuaD and pPT7hasAtuaDgtaBpgi into *Bacillus megaterium* or *E. coli*, preferably in *Bacillus megaterium,*
- Selection of hyaluronic acid-secreting cells by xylose gradient for *Bacillus megaterium* or IPTG gradient for *E. coli,*
- Selection of stable, viable, replicant and secreting high amounts of HA cells.

A further object of the invention is therefore a system for the selection of transfected, secreting, viable cells: the IPTG gradient allows the selection of transfected, viable cells, capable of replication and above all, secreting HA with high yields.

The present invention will be now disclosed by way of example but not of limitation, according to preferred embodiments with particular reference to the attached figures, wherein:

FIG. **1** shows a comparison in plates between the growth of cells *E. coli* TOP10, incorporating plasmid pHT01 (control) and cells *E. coli* TOP10, incorporating pBS5 (hasA+ tuaD);

FIG. **2** shows the gel analysis of the expression of gene tuaD in *E. coli BL*21 DE3;

FIG. **3** shows the analysis in gel electrophoresis of the constitutive expression of hyaluronan synthase (Street) in *E. coli*; the encoded protein designated SeHAS is 417 amino acids long (calculated molecular weight 47,778; calculated PI 9.1) and is the smallest member of the HAS family identified thus far; the enzyme migrates anomalously fast in SDS polyacrylamide gel electrophoresis (about 42000 Da);

FIG. **4** shows the plasmid map pPT7 comprising the promoter and the terminator of T7 RNA polymerase of bacteriophage T7; the replication origin of *Coli* and *Megaterium*; ampicillin resistance gene; tetracycline resistance gene;

FIG. **5** shows the restriction map of plasmid PT7hyal;

FIG. **6** shows the analysis by SDS-page of cell lysates of *E. coli* BL21 DE3 to verify the presence of proteins that lead to the synthesis of hyaluronic acid;

FIG. **7** shows the comparison of the production of HA in plate between colonies of *E. coli* BL21 DE3 transformed with the plasmid pPT7 (colony control), pPT7hasAtuaD (colony 6) and pPT7hasAtuaDgtaBpgi (pT7Hyal—Colony 2) after 24 hours of growth at 37° C., in the presence of IPTG;

FIG. **8** shows the results of plating assays for the selection of cells able to express high levels of hyaluronic acid in the presence or absence of IPTG;

FIG. **9** shows the results of plating assays in the presence of IPTG to test the degree of survival of cells capable of producing HA;

FIG. **10** shows the carbazole analysis of the precipitates of HA in the test tube.

The following examples describe the various steps required for the embodiment of the invention, by way of example but not of limitation.

Example 1

Cloning of the tuaD Gene (UDP-Glucose Dehydrogenase) from *Bacillus subtilis*

The sequence of the tuaD gene, which is 9300 bp long in *B. subtilis*, is present in the databases under the access number AF015609 in the system which codes for the teichuronic acid operon and comprises eight genes, tuaABCDEFGH. In the present case the gene of interest tuaD falls between bases 3582-4984 bp. Software analysis for restriction enzymes indicates that the restriction sites ClaI, EcoRI, PstI, HindIII and SphI are present, and therefore cannot be used for cloning. The start codon is not a methionine but a valine; in the present invention, it was replaced with the codon for methionine, which is much more efficient in the transduction of the protein. Two oligonucleotide primers with the following sequence were used to recover this sequence:

(SEQ ID NO: 3)
5'atgaaaaaatagctgtcattggaacag 3'
and

(SEQ ID NO: 4)
5'ttataaattgtcgttcccaagtct 3'.

The genomic DNA from *B. subtilis* strain 168 (ACTT 23857D-5) was obtained with the Qiagen extraction kit. With 32 cycles of PCR, using DNA from *B. subtilis* as template and the two said oligonucleotides, an amplificate of the expected molecular weight was obtained. The amplificate obtained was tested for the presence of restriction enzyme EcoRI. After cutting with this enzyme in 1% agarose gel, two bands of DNA weighing 470 bp and 920 bp were present, which correspond to those expected. To clone the tuaD gene in an expression vector, two other oligonucleotides with the following sequence were synthesised:

(SEQ ID NO: 5)
5'gctggatccatgaaaaaatagctgtcattgg 3'
and

(SEQ ID NO: 6)
5' ctcgctagcttataaattgacgcttcccaag 3'

in order to insert said sequence between the restriction sites BamHI and NheI in the expression vector, plasmid pRSET B (INVITROGEN).

A Shine-Dalgarno (SD) sequence needs to be introduced into gene tuaD upstream of the 5' end of the gene to allow efficient recognition by the bacterial RNA polymerase. For this purpose the DNA was amplified with the following oligonucleotide primers:

(SEQ ID NO: 7)
5' cgacatatgaaaaaatagctgtcattgg 3'

-continued

and

(SEQ ID NO: 8)
5' ctcgctagcttataaattgacgcttcccaag 3'.

Two restriction sites NdeI and NheI are present in said primers at 5', which allow their cloning in vector pRSET B between the same sites. A sequence SD, consequently present upstream of restriction site NdeI of plasmid pRSET B, is particularly efficient and necessary for the RNA polymerase in order to synthesise the protein. Restriction site XbaI, which will be required for the subsequent clonings, is also present even before said sequence. The vector created, pRSET B, was therefore called pRSEtuaD.

Thus in this plasmid, the sequence coding for tuaD falls between restriction sites NdeI and NheI; restriction site XbaI, which is necessary for the subsequent cloning, is present before and upstream of said plasmid, and other restriction sites, including BamHI--BglII--XhoI, are present behind the tuaD gene.

The diagram below summarises the sites of interest present in plasmid pRSEtuaD

XbaI--NdeI----------tuaD----------NheI--
BamHI--BglI-XhoI

The plasmid described is an expression vector functioning not only in *B. megaterium* but also in *E. coli*, because the gene is under the control of T7 promoter of bacteriophage T7; if it is transformed into bacterial cells BL21 DE3, which are able to transcribe T7 RNA polymerase, it therefore enables them to express the tuaD gene. After induction with 1 mM of IPTG the cells in *E. coli* are able to produce the protein of the expected molecular weight, but not hyaluronic acid. The construction is particularly efficient because the level of expression is very high. The sizes of the colonies which carry plasmid pRSEtuaD are tiny compared with the control cells (FIG. **1**), which demonstrates the toxicity of the tuaD gene. This cloning is difficult precisely because it is apparently difficult for the colonies to grow; the particularly high level of enzyme UDP-glucose dehydrogenase probably drains the cell glucose because it is required for the formation of the hyaluronic acid precursor. The cells in which the synthesis of tuaD is induced with IPTG are therefore no longer able to survive for a long time, so the gene product is toxic.

In conclusion, the tuaD gene was isolated and cloned in a plasmid, and the sequence proved correct. The gene expressed in *E. coli* is able to produce a protein of the expected molecular weight (54 kDa, FIG. **2**), which is toxic to the cell. However, these cells are unable to produce significant quantities of hyaluronic acid, as hyaluronan synthase (hasA) is lacking.

Example 2

Cloning of the hasA (Hyaluronan Synthase) Gene from *Streptococcus zooepidemicus*

The gene sequence for hyaluronan synthase is present in the databases under the access number AY173078, and is 3552 bp long; the sequence coding for the protein is between bases 1 and 1254. The restriction sites HindIII and StuI are present in this sequence, and therefore cannot be used for cloning, but can be used to verify the cloning. Two oligonucleotides for use with PCR were designed and synthesised to recover the coding sequence:

(SEQ ID NO: 9)
5'atgagaacattaaaaaacctcataac 3'
and

(SEQ ID NO: 10)
5'taataatttttacgtgttccccag 3'

The genomic DNA from the bacterium *Streptococcus zooepidemicus* was recovered with the Qiagen extraction kit. The 1254 bp coding sequence was recovered with PCR. The expected amplificate of the correct dimensions was controlled with restriction enzyme HindIII, and gave rise to two bands of approx. 100 bp and 1150 bp which correspond to the expected cut.

Example 3

Construction of the Plasmid pGEM4hasA

Two other oligonucleotides with the following sequence were created to clone the hasA sequence in plasmid pGEM4Z:

(SEQ ID NO: 11)
5' ggaggatccatgagaacattaaaaaacctcat 3'
and

(SEQ ID NO: 12)
5' cagtctagattataataatttttacgtgtcc 3'

The BamHI restriction site was created in the first oligonucleotide close to 5', and the XbaI restriction site was created in the second oligonucleotide, again at 5'. The amplificate obtained through these two oligonucleotides was cloned between restriction sites BamHI and XbaI in plasmid pGEM4Z (PROMEGA) between the same sites to give plasmid pGEM4hasA.

The DNA sequence between said two restriction sites was analysed with an ABI 7000 sequencer, proved correct, and is identical to the one published.

HindIII-BamHI----------hasA----------XbaI-SalI

The plasmid was checked for expression of the recombinant protein in *E. coli* and presented a molecular weight of approx. 42 kDa, which agrees with the weight reported for the protein in the literature, although it has a theoretical molecular weight of 47.778 kDa (FIG. **3**).

The cloning of hasA from *streptococcus* was therefore also demonstrated in terms of protein expression. The plasmid is unable to produce significant quantities of hyaluronic acid because it lacks the tuaD gene.

Example 4

Construction of a Plasmid with the tuaD Gene Following hasA

With this construction, the hasA gene is placed in tandem with the tuaD gene. For this purpose, plasmid pGEM4hasA, which already contains the hasA gene, is used as vector. The plasmid was cut with XbaI and SalI, and the tuaD gene sequence from plasmid pRSEtuaD was cut with XbaI and XhoI and cloned in the same sites (Xho I and SalI are compatible)

pGEM4hasA

HindIII-BamHI----------hasA----------XbaI-SalI

pRSE tuaD

XbaI--NdeI----------tuaD----------NheI-
BamHI--BglI-XhoI

the following final sequence being obtained:

HindIII-BamHI---------hasA----------XbaI--NdeI---------tuaD----------NheI-BamHI--BglI-XhoI

Example 5

Cloning of the hasA-tuaD Gene in the Plasmid pPT7 for *B. megaterium*

This plasmid pPT7 (MoBiTec) contains two origins of replication, one for *E. coli* and one for *B. megaterium*, and can therefore be propagated in both bacteria. It also contains resistance to the antibiotics ampicillin and tetracycline, which can be used for *E. coli* and *B. megaterium* respectively, and the recognition sequence for T7 RNA polymerase, namely the promoter dependent on T7 RNA polymerase of bacteriophage T7 followed by its terminator.

The plasmid contains restriction site BsrGI with the sequence tgtaca a few bases after the Shine-Dalgarno sequence, and a site BamHI (ggatcc) after the initial methionine. Two oligonucleotides were synthesised for the cloning so as to create the following two restriction sites at the end:

(SEQ ID NO: 13)
5'GCTTGTACATGAGAACATTAAAAAACCTCA 3'

(SEQ ID NO: 14)
5'AGGGATCCTTATAAATTGACGCTTCCCAAG 3'

i.e. BsrGI and BamHI upstream and downstream of genes hasA and tuaD respectively. The 2698 bp amplificate obtained was cut with the restriction enzymes BsrGI and BamHI and cloned in the same restriction sites as plasmid pPT7 to obtain plasmid pPT7hasAtuaD (FIG. **4**).

The complete sequence of this plasmid, called pPT7hasAtuaD, was analysed, and is set out below:

```
   0  CTTTTTAGGTTCTAAATCGTGTTTTTCTTGGAATTGTGCTGTTTTATCCTTTACCTTGTC
  60  TACAAACCCCTTAAAAACGTTTTTAAAGGCTTTTAAGCCGTCTGTACGTTCCTTAAGGCG
 120  AAATTAATACGACTCACTATAGGGAGACCACAACGGTTTCCCGAATATTAATTAACCAAG
                Bsp1407I
 180  GAGGTGAAATGTACAATGAGAACATTAAAAAACCTCATAACTGTTGTGGCCTTTAGTATT
   1                M  R  T  L  K  N  L  I  T  V  V  A  F  S  I
HindIII
 240  TTTTGGGTACTGTTGATTTACGTCAATGTTTATCTCTTTGGTGCTAAAGGAAGCTTGTCA
   1  F  W  V  L  L  I  Y  V  N  V  Y  L  F  G  A  K  G  S  L  S
 300  ATTTATGGCTTTTTGCTGATAGCTTACCTATTAGTCAAAATGTCCTTATCCTTTTTTTAC
   1  I  Y  G  F  L  L  I  A  Y  L  L  V  K  M  S  L  S  F  F  Y
 360  AAGCCATTTAAGGGAAGGGCTGGGCAATATAAGGTTGCAGCCATTATTCCCTCTTATAAC
   1  K  P  F  K  G  R  A  G  Q  Y  K  V  A  A  I  I  P  S  Y  N
 420  GAAGATGCTGAGTCATTGCTAGAGACCTTAAAAAGTGTTCAGCAGCAAACCTATCCCCTA
   1  E  D  A  E  S  L  L  E  T  L  K  S  V  Q  Q  Q  T  Y  P  L
 480  GCAGAAATTTATGTTGTTGACGATGGAAGTGCTGATGAGACAGGTATTAAGCGCATTGAA
   1  A  E  I  Y  V  V  D  D  G  S  A  D  E  T  G  I  K  R  I  E
 540  GACTATGTGCGTGACACTGGTGACCTATCAAGCAATGTCATTGTTCACCGGTCAGAAAAA
   1  D  Y  V  R  D  T  G  D  L  S  S  N  V  I  V  H  R  S  E  K
 600  AATCAAGGAAAGCGTCATGCACAGGCCTGGGCCTTTGAAAGATCAGACGCTGATGTCTTT
   1  N  Q  G  K  R  H  A  Q  A  W  A  F  E  R  S  D  A  D  V  F
 660  TTGACCGTTGACTCAGATACTTATATCTACCCTGATGCTTTAGAGGAGTTGTTAAAAACC
   1  L  T  V  D  S  D  T  Y  I  Y  P  D  A  L  E  E  L  L  K  T
 720  TTTAATGACCCAACTGTTTTTGCTGCGACGGGTCACCTTAATGTCAGAAATAGACAAACC
   1  F  N  D  P  T  V  F  A  A  T  G  H  L  N  V  R  N  R  Q  T
 780  AATCTCTTAACACGCTTGACAGATATTCGCTATGATAATGCTTTTGGCGTTGAACGAGCT
   1  N  L  L  T  R  L  T  D  I  R  Y  D  N  A  F  G  V  E  R  A
 840  GCCCAATCCGTTACAGGTAATATTCTCGTTTGCTCAGGCCCGCTTAGCGTTTACAGACGC
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```
   1  A  Q  S  V  T  G  N  I  L  V  C  S  G  P  L  S  V  Y  R  R
 900  GAGGTGGTTGTTCCTAACATAGATAGATACATCAACCAGACCTTCCTGGGTATTCCTGTA
   1  E  V  V  V  P  N  I  D  R  Y  I  N  Q  T  F  L  G  I  P  V
 960  AGTATCGGTGATGACAGGTGCTTGACCAACTATGCAACTGATTTAGGAAAGACTGTTTAT
   1  S  I  G  D  D  R  C  L  T  N  Y  A  T  D  L  G  K  T  V  Y
1020  CAATCCACTGCTAAATGTATTACAGATGTTCCTGACAAGATGTCTACTTACTTGAAGCAG
   1  Q  S  T  A  K  C  I  T  D  V  P  D  K  M  S  T  Y  L  K  Q
1080  CAAAACCGCTGGAACAAGTCCTTCTTTAGAGAGTCCATTATTTCTGTTAAGAAAATCATG
   1  Q  N  R  W  N  K  S  F  F  R  E  S  T  I  S  V  K  K  I  M
1140  AACAATCCTTTTGTAGCCCTATGGACCATACTTGAGGTGTCTATGTTTATGATGCTTGTT
   1  N  N  P  F  V  A  L  T  I  L  E  E  V  S  M  F  M  M  L  V
1200  TATTCTGTGGTGGATTTCTTTGTAGGCAATGTCAGAGAATTTGATTGGCTCAGGGTTTTG
   1  Y  S  V  V  D  F  F  V  G  N  V  R  E  F  D  W  L  R  V  L
1260  GCCTTTCTGGTGATTATCTTCATTGTTGCTCTTTGTCGTAATATTCACTATATGCTTAAG
   1  A  F  L  V  I  I  F  I  V  A  L  C  R  N  I  H  Y  M  L  K
1320  CACCCGCTGTCCTTCTTGTTATCTCCGTTTTATGGGGTACTGCTTTGTTTGTCCTACAGC
   1  H  P  L  S  F  L  L  S  P  F  Y  G  V  L  L  C  L  S  Y  S
1380  CCTTGAAATTGTATTCTCTTTTTACTATTAGAAATGCTGACTGGGGAACACGTAAAAAAT
   1  P

            XbaI                                          NdeI
1440  TATTATAATCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACATATGAAAAAAA
   3                                                    M  K  K  I
1500  TAGCTGTCATTGGAACAGGTTATGTAGGACTCGTATCAGGCACTTGCTTTGCGGAGATCG
   3    A  V  I  G  T  G  Y  V  G  L  V  S  G  T  C  F  A  E  I  G

                        EcoRV  ClaI
1560  GCAATAAAGTTGTTTGCTGTGATATCGATGAATCAAAAATCAGAAGCCTGAAAAATGGGG
   3    N  K  V  V  C  C  D  I  D  E  S  K  I  R  S  L  K  N  G  V
1620  TAATCCCAATCTATGAACCAGGGCTTGCAGACTTAGTTGAAAAAAATGTGCTGGATCAGC
   3    I  P  I  Y  E  P  G  L  A  D  L  V  E  K  N  V  L  D  Q  R

                          EcoRV
1680  GCCTGACCTTTACGAACGATATCCCGTCTGCCATTCGGGCCTCAGATATTATTTATATTG
   3    L  T  F  T  N  D  I  P  S  A  I  R  A  S  D  I  I  Y  I  A
1740  CAGTCGGAACGCCTATGTCCAAAACAGGTGAAGCTGATTTAACGTACGTCAAAGCGGCGG
   3    V  G  T  P  M  S  K  T  G  E  A  D  L  T  Y  V  K  A  A  A
1800  CGAAAACAATCGGTGAGCATCTTAACGGCTACAAAGTGATCGTAAATAAAAGCACAGTCC
   3    K  T  I  G  E  H  L  N  G  Y  K  V  I  V  N  K  S  T  V  P
1860  CGGTTGGAACAGGGAAACTGGTGCAATCTATCGTTCAAAAAGCCTCAAAGGGGAGATACT
   3    V  G  T  G  K  L  V  Q  S  I  V  Q  K  A  S  K  G  R  Y  S

                              EcoRI
1920  CATTTGATGTTGTATCTAACCCTGAATTCCTTCGGGAAGGGTCAGCGATTCATGACACGA
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   3    F  D  V  V  S  N  P  E  F  L  R  E  G  S  A  I  H  D  T  M

1980    TGAATATGGAGCGTGCCGTGATTGGTTCAACAAGTCATAAAGCCGCTGCCATCATTGAGG

   3    N  M  E  R  A  V  I  G  S  T  S  H  K  A  A  A  I  I  E  E

2040    AACTTCATCAGCCATTCCATGCTCCTGTCATTAAAACAAACCTAGAAAGTGCAGAAATGA

   3    L  H  Q  P  F  H  A  P  V  I  K  T  N  L  E  S  A  E  M  I

EcoRV

2100    TTAAATACGCCGCGAATGCATTTCTGGCGACAAAGATTTCCTTTATCAACGATATCGCAA

   3    K  Y  A  A  N  A  F  L  A  T  K  I  S  F  I  N  D  I  A  N

2160    ACATTTGTGAGCGAGTCGGCGCAGACGTTTCAAAAGTTGCTGATGGTGTTGGTCTTGACA

   3    I  C  E  R  V  G  A  D  V  S  K  V  A  D  G  V  G  L  D  S

2220    GCCGTATCGGCAGAAAGTTCCTTAAAGCTGGTATTGGATTCGGCGGTTCATGTTTTCCAA

   3    R  I  G  R  K  F  L  K  A  G  I  G  F  G  G  S  C  F  P  K

2280    AGGATACAACCGCGCTGCTTCAAATCGCAAAATCGGCAGGCTATCCATTCAAGCTCATCG

   3    D  T  T  A  L  L  Q  I  A  K  S  A  G  Y  P  F  K  L  I  E

2340    AAGCTGTCATTGAAACGAACGAAAAGCAGCGTGTTCATATTGTAGATAAACTTTTGACTG

   3    A  V  I  E  T  N  E  K  Q  R  V  H  I  V  D  K  L  L  T  V

2400    TTATGGGAAGCGTCAAAGGGAGAACCATTTCAGTCCTGGGATTAGCCTTCAAACCGAATA

   3    M  G  S  V  K  G  R  T  I  S  V  L  G  L  A  F  K  P  N  T

                                                    PstI

2460    CGAACGATGTGAGATCCGCTCCAGCGCTTGATATTATCCCAATGCTGCAGCAGCTGGGCG

   3    N  D  V  R  S  A  P  A  L  D  I  I  P  M  L  Q  Q  L  G  A

HindIII

2520    CCCATGTAAAAGCATACGATCCGATTGCTATTCCTGAAGCTTCAGCGATCCTTGGCGAAC

   3    H  V  K  A  Y  D  P  I  A  I  P  E  A  S  A  I  L  G  E  Q

                                                          SphI

2580    AGGTCGAGTATTACACAGATGTGTATGCTGCGATGGAAGACACTGATGCATGCCTGATTT

   3    V  E  Y  Y  T  D  V  Y  A  A  M  E  D  T  D  A  C  L  I  L

2640    TAACGGATTGGCCGGAAGTGAAAGAAATGGAGCTTGTAAAAGTGAAAACCCTCTTAAAAC

   3    T  D  W  P  E  V  K  E  M  E  L  V  K  V  K  T  L  L  K  Q

2700    AGCCAGTCATCATTGACGGCAGAAATTTATTTTCACTTGAAGAGATGCAGGCAGCCGGAT

   3    P  V  I  I  D  G  R  N  L  F  S  L  E  E  M  Q  A  A  G  Y

2760    ACATTTATCACTCTATCGGCCGTCCCGCTGTTCGGGGAACGGAACCCTCTGACAAGTATT

   3    I  Y  H  S  I  G  R  P  A  V  R  G  T  E  P  S  D  K  Y  F

BamHI

2820    TTCCGGGCTTGCCGCTTGAAGAATTGGCTAAAGACTTGGGAAGCGTCAATTTATAAGGAT

   3    P  G  L  P  L  E  E  L  A  K  D  L  G  S  V  N  L

                SphI

                                                          (SEQ ID NO: 1)
2880    CCGGCCGCATGCCGGCTAATCGCGACCGGTTAACTAGCATAACCCCTTGGGGCCTCTAAA

2940    CGGGTCTTGAGGGGTTTTTTGCTAAAGGAGGAACTATATCCGGTCCAAGAATTGGAGCCA

3000    ATCAATTCTTGCGGAGAACTGTGAATGCGCAAACCAACCCTTGGCAGAACATATCCATCG
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3060  CGTCCGCCATCTCCAGCAGCCGCACGCGGCGCATCTCGGGCCGCGTTGCTGGCGTTTTTC

3120  CATAGGCTCCGCCCCCCTGACGAGCATCACAAAAATCGACGCTCAAGTCAGAGGTGGCGA

3180  AACCCGACAGGACTATAAAGATACCAGGCGTTTCCCCCTGGAAGCTCCCTCGTGCGCTCT

3240  CCTGTTCCGACCCTGCCGCTTACCGGATACCTGTCCGCCTTTCTCCCTTCGGGAAGCGTG

3300  GCGCTTTCTCATAGCTCACGCTGTAGGTATCTCAGTTCGGTGTAGGTCGTTCGCTCCAAG

3360  CTGGGCTGTGTGCACGAACCCCCCGTTCAGCCCGACCGCTGCGCCTTATCCGGTAACTAT

3420  CGTCTTGAGTCCAACCCGGTAAGACACGACTTATCGCCACTGGCAGCAGCCACTGGTAAC

3480  AGGATTAGCAGAGCGAGGTATGTAGGCGGTGCTACAGAGTTCTTGAAGTGGTGGCCTAAC

3540  TACGGCTACACTAGAAGGACAGTATTTGGTATCTGCGCTCTGCTGAAGCCAGTTACCTTC

3600  GGAAAAAGAGTTGGTAGCTCTTGATCCGGCAAACAAACCACCGCTGGTAGCGGTGGTTTT

3660  TTTGTTTGCAAGCAGCAGATTACGCGCAGAAAAAAAGGATCTCAAGAAGATCCTTTGATC

3720  TTTTCTACGGGGTCTGACGCTCAGTGGAACGAAAACTCACGTTAAGGGATTTTGGTCATG

3780  AGATTATCAAAAAGGATCTTCACCTAGATCCTTTTAAATTAAAAATGAAGTTTTAAATCA

3840  ATCTAAAGTATATATGAGTAAACTTGGTCTGACAGTTACCAATGCTTAATCAGTGAGGCA

3900  CCTATCTCAGCGATCTGTCTATTTCGTTCATCCATAGTTGCCTGACTCCCCGTCGTGTAG

3960  ATAACTACGATACGGGAGGGCTTACCATCTGGCCCCAGTGCTGCAATGATACCGCGAGAC

4020  CCACGCTCACCGGCTCCAGATTTATCAGCAATAAACCAGCCAGCCGGAAGGGCCGAGCGC

4080  AGAAGTGGTCCTGCAACTTTATCCGCCTCCATCCAGTCTATTAATTGTTGCCGGGAAGCT

PstI

4140  AGAGTAAGTAGTTCGCCAGTTAATAGTTTGCGCAACGTTGTTGCCATTGCTGCAGGCATC

                    Hpy99I

4200  GTGGTGTCACGCTCGTCGTTTGGTATGGCTTCATTCAGCTCCGGTTCCCAACGATCAAGG

4260  CGAGTTACATGATCCCCCATGTTGTGCAAAAAAGCGGTTAGCTCCTTCGGTCCTCCGATC

4320  GTTGTCAGAAGTAAGTTGGCCGCAGTGTTATCACTCATGGTTATGGCAGCACTGCATAAT

                                    ScaI

4380  TCTCTTACTGTCATGCCATCCGTAAGATGCTTTTCTGTGACTGGTGAGTACTCAACCAAG

4440  TCATTCTGAGAATAGTGTATGCGGCGACCGAGTTGCTCTTGCCCGGCGTCAACACGGGAT

4500  AATACCGCGCCACATAGCAGAACTTTAAAAGTGCTCATCATTGGAAAACGTTCTTCGGGG

4560  CGAAAACTCTCAAGGATCTTACCGCTGTTGAGATCCAGTTCGATGTAACCCACTCGTGCA

4620  CCCAACTGATCTTCAGCATCTTTTACTTTCACCAGCGTTTCTGGGTGAGCAAAAACAGGA

4680  AGGCAAAATGCCGCAAAAAAGGGAATAAGGGCGACACGGAAATGTTGAATACTCATACTC

4740  TTCCTTTTTCAATATTATTGAAGCATTTATCAGGGTTATTGTCTCATGAGCGGATACATA

4800  TTTGAATGTATTTAGAAAAATAAACAAATAGGGGTTCCGCGCACATTTCCCCGAAAAGTG

4860  CCACCTGACGTCTAAGAAACCATTATTATCATGACATTAACCTATAAAAATAGGCGTATC

EcoRI

4920  ACGAGGCCCTTTCGTCTTCAAGAATTCCTGTTATAAAAAAAGGATCAATTTTGAACTCTC

4980  TCCCAAAGTTGATCCCTTAACGATTTAGAAATCCCTTTGAGAATGTTTATATACATTCAA

   —

5040  GGTAACCAGCCAACTAATGACAATGATTCCTGAAAAAAGTAATAACAAATTACTATACAG

5100  ATAAGTTGACTGATCAACTTCCATAGGTAACAACCTTTGATCAAGTAAGGGTATGGATAA

5160  TAAACCACCTACAATTGCAATACCTGTTCCCTCTGATAAAAAGCTGGTAAAGTTAAGCAA
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5220  ACTCATTCCAGCACCAGCTTCCTGCTGTTTCAAGCTACTTGAAACAATTGTTGATATAAC

5280  TGTTTTGGTGAACGAAAGCCCACCTAAAACAAATACGATTATAATTGTCATGAACCATGA

5340  TGTTGTTTCTAAAAGAAAGGAAGCAGTTAAAAAGCTAACAGAAAGAAATGTAACTCCGAT

5400  GTTTAACACGTATAAAGGACCTCTTCTATCAACAAGTATCCCACCAATGTAGCCGAAAAT

                                                        ScaI

5460  AATGACACTCATTGTTCCAGGGAAAATAATTACACTTCCGATTTCGGCAGTACTTAGCTG

5520  GTGAACATCTTTCATCATATAAGGAACCATAGAGACAAACCCTGCTACTGTTCCAAATAT

5580  AATTCCCCCACAAAGAACTCCAATCATAAAAGGTATATTTTTCCCTAATCCGGGATCAAC

5640  AAAAGGATCTGTTACTTTCCTGATATGTTTTACAAATATCAGGAATGACAGCACGCTAAC

5700  GATAAGAAAAGAAATGCTATATGATGTTGTAAACAACATAAAAAATACAATGCCTACAGA

                    EcoRV

5760  CATTAGTATAATTCCTTTGATATCAAAATGACCTTTTATCCTTACTTCTTTCTTTAATAA

5820  TTTCATAAGAAACGGAACAGTGATAATTGTTATCATAGGAATGAGTAGAAGATAGGACCA

5880  ATGAATATAATGGGCTATCATTCCACCAATCGCTGGACCGACTCCTTCTCCCATGGCTAC

          ClaI

5940  TATCGATCCAATAAGACCAAATGCTTTACCCCTATTTTCCTTTGGAATATAGCGCGCAAC

6000  TACAACCATTACGAGTGCTGGAAATGCAGCTGCACCAGCCCCTTGAATAAAACGAGCCAT

6060  AATAAGTAAGGAAAAGAAAGAATGGCCAACAAACCCAATTACCGACCCGAAACAATTTAT

6120  TATAATTCCAAATAGGAGTAACCTTTTGATGCCTAATTGATCAGATAGCTTTCCATATAC

6180  AGCTGTTCCAATGGAAAAGGTTAACATAAAGGCTGTGTTCACCCAGTTTGTACTCGCAGG

6240  TGGTTTATTAAAATCATTTGCAATATCAGGTAATGAGACGTTCAAAACCATTTCATTTAA

6300  TACGCTAAAAAAAGATAAAATGCAAAGCCAAATTAAAATTTGGTTGTGTCGTAAATTCGA

6360  TTGTGAATAGGATGTATTCACATTTCACCCTCCAATAATGAGGGCAGACGTAGTTTATAG

6420  GGTTAATGATACGCTTCCCTCTTTTAATTGAACCCTGTTACATTCATTACACTTCATAAT

6480  TAATTCCTCCTAAACTTGATTAAAACATTTTACCACATATAAACTAAGTTTTAAATTCAG

6540  TATTTCATCACTTATACAACAATATGGCCCGTTTGTTGAACTACTCTTTAATAAAATAAT

6600  TTTTCCGTTCCCAATTCCACATTGCAATAATAGAAAATCCATCTTCATCGGCTTTTTCGT

6660  CATCATCTGTATGAATCAAATCGCCTTCTTCTGTGTCATCAAGGTTTAATTTTTTATGTA

6720  TTTCTTTTAACAAACCACCATAGGAGATTAACCTTTTACGGTGTAAACCTTCCTCCAAAT

6780  CAGACAAACGTTTCAAATTCTTTTCTTCATCATCGGTCATAAAATCCGTATCCTTTACAG

6840  GATATTTTGCAGTTTCGTCAATTGCCGATTGTATATCCGATTTATATTTATTTTTCGGTC

6900  GAATCATTTGAACTTTTACATTTGGATCATAGTCTAATTTCATTGCCTTTTTCCAAAATT

6960  GAATCCATTGTTTTTGATTCACGTAGTTTTCTGTATTCTTAAAATAAGTTGGTTCCACAC

7020  ATACCAATACATGCATGTGCTGATTATAAGAATTATCTTTATTATTTATTGTCACTTCCG

7080  TTGCACGCATAAAACCAACAAGATTTTTATTAATTTTTTTATATTGCATCATTCGGCGAA

7140  ATCCTTGAGCCATATCTGACAAACTCTTATTTAATTCTTCGCCATCATAAACATTTTTAA

7200  CTGTTAATGTGAGAAACAACCAACGAACTGTTGGCTTTTGTTTAATAACTTCAGCAACAA

7260  CCTTTTGTGACTGAATGCCATGTTTCATTGCTCTCCTCCAGTTGCACATTGGACAAAGCC

7320  TGGATTTACAAAACCACACTCGATACAACTTTCTTTCGCCTGTTTCACGATTTTGTTTAT

7380  ACTCTAATATTTCAGCACAATCTTTTACTCTTTCAGCCTTTTTAAATTCAAGAATATGCA
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7440  GAAGTTCAAAGTAATCAACATTAGCGATTTTCTTTTCTCTCCATGGTCTCACTTTTCCAC

7500  TTTTTGTCTTGTCCACTAAAACCCTTGATTTTTCATCTGAATAAATGCTACTATTAGGAC

7560  ACATAATATTAAAAGAAACCCCCATCTATTTAGTTATTTGTTTGGTCACTTATAACTTTA

7620  ACAGATGGGGTTTTTCTGTGCAACCAATTTTAAGGGTTTTCAATACTTTAAAACACATAC

7680  ATACCAACACTTCAACGCACCTTTCAGCAACTAAAATAAAAATGACGTTATTTCTATATG

7740  TATCAAGATAAGAAAGAACAAGTTCAAAACCATCAAAAAAAGACACCTTTTCAGGTGCTT

7800  TTTTTATTTTATAAACTCATTCCCTGATCTCGACTTCGTTCTTTTTTTACCTCTCGGTTA

7860  TGAGTTAGTTCAAATTCGTT
```

The plasmid has a molecular weight of 7880 bp and contains the various genes responsible for hyaluronic acid synthesis under the control of strong T7 promoter of bacteriophage T7. The hasA synthase sequence from *Streptococcus equi* falls between bases 196 and 1383, and that of the tuaD gene between bases 1430 and 2873.

Example 6

Cloning of the gtaB Gene (UDP-Glc Pyrophosphorylase)

The gtaB gene from *Bacillus Subtilis* was recovered from the bacterial genome as above, and through two oligonucleotides having the following sequence:

(SEQ ID NO: 15)
5'ATGTCTAGAATAATAAGGAAGGTGCCTTTTAAATGAA 3'

(SEQ ID NO: 16)
5'CTCTCGAGCTAGCTTAGATTTCTTCTTTGTTTAGTAAAG 3'

The amplified product of 925 bp was cut with XbaI and XhoI and cloned in plasmid pGEM4hasA in the same restriction sites; plasmid pGEMhasA-gtaB is obtained in this way.

Example 7

Cloning of the Pgi Gene from *Bacillus subtilis* in Plasmid pRSET B

The pgi gene (glucose 6 phosphate isomerase, also called phosphoglucoisomerase pgi, corresponding to hasE from *S. zooepidemicus*) was recovered from the bacterial genome as described above with these two oligonucleotides

(SEQ ID NO: 17)
5'TACATATGACGCATGTACGCTTGACTACTCCAAAAG 3'

(SEQ ID NO: 18)
5'ATGCTAGCTCATTTATAATCTTCCAGACGTTTTTCAAG 3'

and PCR, and cloned after cutting with restriction enzymes NdeI and NheI in plasmid pRSETB between the same restriction sites. Plasmid pRSEpgi is obtained in this way. It places the pgi gene under the control of a T7 promoter, and when it is transferred to cells of *E. coli* BL21 DE3 it produces the protein of the expected molecular weight. This plasmid was cut with XbaI and PstI, and the 1340 bp fragment was cloned in plasmid pGEMhasA-gtaB between sites NheI and PstI. Restriction site Xba, like NheI, is lost after cloning. In this way the pgi gene is placed behind the gtaB gene. The plasmid, called pGEM hasA-gtaB-pgi, was cut with XbaI and XhoI, and the fragment which contains the sequences coding for gtaB and pgi was cloned in plasmid pRSEtuaD between the same sites. The plasmid obtained was called pRSEtuaD-gtaB-pgi.

The latter was cut with XbaI and BamHI and the fragment which contains the sequence coding for tuaD, gtaB and pgi was cloned in plasmid pPT7hasAtuaD between the same sites to obtain plasmid pPT7hasAtuaDgtaBpgi, which we will call pT7hyal.

The sequence of plasmid pT7hyal is shown below

```
   0  CTTTTTAGGTTCTAAATCGTGTTTTTCTTGGAATTGTGCTGTTTTATCCTTTACCTTGTC

  60  TACAAACCCCTTAAAAACGTTTTTAAAGGCTTTTAAGCCGTCTGTACGTTCCTTAAGGCG

 120  AAATTAATACGACTCACTATAGGGAGACCACAACGGTTTCCCGAATATTAATTAACCAAG

                Bsp1407I

 180  GAGGTGAAATGTACAATGAGAACATTAAAAAACCTCATAACTGTTGTGGCCTTTAGTATT

   1                M  R  T  L  K  N  L  I  T  V  V  A  F  S  I

HindIII

 240  TTTTGGGTACTGTTGATTTACGTCAATGTTTATCTCTTTGGTGCTAAAGGAAGCTTGTCA

   1  F  W  V  L  L  I  Y  V  N  V  Y  L  F  G  A  K  G  S  L  S

 300  ATTTATGGCTTTTTGCTGATAGCTTACCTATTAGTCAAAATGTCCTTATCCTTTTTTTAC

   1  I  Y  G  F  L  L  I  A  Y  L  L  V  K  M  S  L  S  F  F  Y
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360  AAGCCATTTAAGGGAAGGGCTGGGCAATATAAGGTTGCAGCCATTATTCCCTCTTATAAC
  1  K  P  F  K  G  R  A  G  Q  Y  K  V  A  A  I  I  P  S  Y  N
420  GAAGATGCTGAGTCATTGCTAGAGACCTTAAAAAGTGTTCAGCAGCAAACCTATCCCCTA
  1  E  D  A  E  S  L  L  E  T  L  K  S  V  Q  Q  Q  T  Y  P  L
480  GCAGAAATTTATGTTGTTGACGATGGAAGTGCTGATGAGACAGGTATTAAGCGCATTGAA
  1  A  E  I  Y  V  V  D  D  G  S  A  D  E  T  G  I  K  R  I  E
540  GACTATGTGCGTGACACTGGTGACCTATCAAGCAATGTCATTGTTCACCGGTCAGAAAAA
  1  D  Y  V  R  D  T  G  D  L  S  S  N  V  I  V  H  R  S  E  K
600  AATCAAGGAAAGCGTCATGCACAGGCCTGGGCCTTTGAAAGATCAGACGCTGATGTCTTT
  1  N  Q  G  K  R  H  A  Q  A  W  A  F  E  R  S  D  A  D  V  F
660  TTGACCGTTGACTCAGATACTTATATCTACCCTGATGCTTTAGAGGAGTTGTTAAAAACC
  1  L  T  V  D  S  D  T  Y  I  Y  P  D  L  E  E  E  L  L  K  T
720  TTTAATGACCCAACTGTTTTTGCTGCGACGGGTCACCTTAATGTCAGAAATAGACAAACC
  1  F  N  D  P  T  V  F  A  A  T  G  H  L  N  V  R  N  R  Q  T
780  AATCTCTTAACACGCTTGACAGATATTCGCTATGATAATGCTTTTGGCGTTGAACGAGCT
  1  N  L  L  T  R  L  T  D  I  R  Y  D  N  A  F  G  V  E  R  A
840  GCCCAATCCGTTACAGGTAATATTCTCGTTTGCTCAGGCCCGCTTAGCGTTTACAGACGC
  1  A  Q  S  V  T  G  N  I  L  V  C  S  G  P  L  S  V  Y  R  R
900  GAGGTGGTTGTTCCTAACATAGATAGATACATCAACCAGACCTTCCTGGGTATTCCTGTA
  1  E  V  V  V  P  N  I  D  R  Y  I  N  Q  T  F  L  G  I  P  V
960  AGTATCGGTGATGACAGGTGCTTGACCAACTATGCAACTGATTTAGGAAAGACTGTTTAT
  1  S  I  G  D  D  R  C  L  T  N  Y  A  T  D  L  G  K  T  V  Y
1020 CAATCCACTGCTAAATGTATTACAGATGTTCCTGACAAGATGTCTACTTACTTGAAGCAG
  1  Q  S  T  A  K  C  I  T  D  V  P  D  K  M  S  T  Y  L  K  Q
1080 CAAAACCGCTGGAACAAGTCCTTCTTTAGAGAGTCCATTATTTCTGTTAAGAAAATCATG
  1  Q  N  R  W  N  K  S  F  F  R  E  S  I  I  S  V  K  K  I  M
1140 AACAATCCTTTTGTAGCCCTATGGACCATACTTGAGGTGTCTATGTTTATGATGCTTGTT
  1  N  N  P  F  V  A  L  W  T  I  L  E  V  S  M  F  M  M  L  V
1200 TATTCTGTGGTGGATTTCTTTGTAGGCAATGTCAGAGAATTTGATTGGCTCAGGGTTTTG
  1  Y  S  V  V  D  F  F  V  G  N  V  R  E  F  D  W  L  R  V  L
1260 GCCTTTCTGGTGATTATCTTCATTGTTGCTCTTTGTCGTAATATTCACTATATGCTTAAG
  1  A  F  L  V  I  I  F  I  V  A  L  C  R  N  I  H  Y  M  L  K
1320 CACCCGCTGTCCTTCTTGTTATCTCCGTTTTATGGGGTACTGCTTTGTTTGTCCTACAGC
  1  H  P  L  S  F  L  L  S  P  F  Y  G  V  L  L  C  L  S  Y  S
1380 CCTTGAAATTGTATTCTCTTTTTACTATTAGAAATGCTGACTGGGGAACACGTAAAAAAT
  1  P

           XbaI                                    NdeI
1440 TATTATAATCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACATATGAAAAAAA
  3                                                M  K  K  I
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1500  TAGCTGTCATTGGAACAGGTTATGTAGGACTCGTATCAGGCACTTGCTTTGCGGAGATCG
   3    A  V  I  G  T  G  Y  V  G  L  V  S  G  T  C  F  A  E  I  G
                         EcoRV
ClaI
1560  GCAATAAAGTTGTTTGCTGTGATATCGATGAATCAAAAATCAGAAGCCTGAAAAATGGGG
   3    N  K  V  V  C  C  D  I  D  E  S  K  I  R  S  L  K  N  G  V
1620  TAATCCCAATCTATGAACCAGGGCTTGCAGACTTAGTTGAAAAAAATGTGCTGGATCAGC
   3    I  P  I  Y  E  P  G  L  A  D  L  V  E  K  N  V  L  D  Q  R
                        EcoRV
1680  GCCTGACCTTTACGAACGATATCCCGTCTGCCATTCGGGCCTCAGATATTATTTATATTG
   3    L  T  F  T  N  D  I  P  S  A  I  R  A  S  D  I  I  Y  I  A
1740  CAGTCGGAACGCCTATGTCCAAAACAGGTGAAGCTGATTTAACGTACGTCAAAGCGGCGG
   3    V  G  T  P  M  S  K  T  G  E  A  D  L  T  Y  V  K  A  A  A
1800  CGAAAACAATCGGTGAGCATCTTAACGGCTACAAAGTGATCGTAAATAAAAGCACAGTCC
   3    K  T  I  G  E  H  L  N  G  Y  K  V  I  V  N  K  S  T  V  P
1860  CGGTTGGAACAGGGAAACTGGTGCAATCTATCGTTCAAAAAGCCTCAAAGGGGAGATACT
   3    V  G  T  G  K  L  V  Q  S  I  V  Q  K  A  S  K  G  R  Y  S
                              EcoRI
1920  CATTTGATGTTGTATCTAACCCTGAATTCCTTCGGGAAGGGTCAGCGATTCATGACACGA
   3    F  D  V  V  S  N  P  E  F  L  R  E  G  S  A  I  H  D  T  M
1980  TGAATATGGAGCGTGCCGTGATTGGTTCAACAAGTCATAAAGCCCTGCCATCATTGAGG
   3    N  M  E  R  A  V  I  G  S  T  S  H  K  A  A  A  I  I  E  E
2040  AACTTCATCAGCCATTCCATGCTCCTGTCATTAAAACAAACCTAGAAAGTGCAGAAATGA
   3    L  H  Q  P  F  H  A  P  V  I  K  T  N  L  E  S  A  E  M  I
EcoRV
2100   TTAAATACGCCGCGAATGCATTTCTGGCGACAAAGATTTCCTTTATCAACGATATCGCAA
   3    K  Y  A  A  N  A  F  L  A  T  K  I  S  F  I  N  D  I  A  N
2160  ACATTTGTGAGCGAGTCGGCGCAGACGTTTCAAAAGTTGCTGATGGTGTTGGTCTTGACA
   3    I  C  E  R  G  A  D  D  V  S  K  V  A  D  G  V  G  L  D  S
2220  GCCGTATCGGCAGAAAGTTCCTTAAAGCTGGTATTGGATTCGGCGGTTCATGTTTTCCAA
   3    R  I  G  R  K  F  L  K  G  I  G  G  F  G  G  S  C  F  P  K
2280  AGGATACAACCGCGCTGCTTCAAATCGCAAAATCGGCAGGCTATCCATTCAAGCTCATCG
   3    D  T  T  A  L  L  Q  I  A  K  S  A  G  Y  P  F  K  L  I  E
2340  AAGCTGTCATTGAAACGAACGAAAAGCAGCGTGTTCATATTGTAGATAAACTTTTGACTG
   3    A  V  I  E  T  N  E  K  Q  R  V  H  I  V  D  K  L  L  T  V
2400  TTATGGGAAGCGTCAAAGGGAGAACCATTTCAGTCCTGGGATTAGCCTTCAAACCGAATA
   3    M  G  S  V  K  G  R  T  I  S  V  L  G  L  A  F  K  P  N  T
                                                    PstI
2460  CGAACGATGTGAGATCCGCTCCAGCGCTTGATATTATCCCAATGCTGCAGCAGCTGGGCG
   3    N  D  V  R  S  A  P  A  L  D  I  I  P  M  L  Q  Q  L  G  A
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HindIII

2520 CCCATGTAAAAGCATACGATCCGATTGCTATTCCTGAAGCTTCAGCGATCCTTGGCGAAC

3 H V K A Y D P I A I P E A S A I L G E Q

SphI

2580 AGGTCGAGTATTACACAGATGTGTATGCTGCGATGGAAGACACTGATGCATGCCTGATTT

3 V E Y Y T D V Y A A M E D T D A C L I L

2640 TAACGGATTGGCCGGAAGTGAAAGAAATGGAGCTTGTAAAAGTGAAAACCCTCTTAAAAC

3 T D W P E V K E M E L V K V K T L L K Q

2700 AGCCAGTCATCATTGACGGCAGAAATTTATTTTCACTTGAAGAGATGCAGGCAGCCGGAT

3 P V I I D G R N L F S L E E M Q A A G Y

2760 ACATTTATCACTCTATCGGCCGTCCCGCTGTTCGGGGAACGGAACCCTCTGACAAGTATT

3 I Y H S I G R P A V R G T E P S D K Y F

2820 TTCCGGGCTTGCCGCTTGAAGAATTGGCTAAAGACTTGGGAAGCGTCAATTTATAAGCTA

3 P G L P L E E L A K D L G S V N L

2880 GAATAATAAGGAAGGTGCCTTTTAAATGAAAAAAGTACGTAAAGCCATAATTCCAGCAGC

2 M K K V R K A I I P A A

2940 AGGCTTAGGAACACGTTTTCTTCCGGCTACGAAAGCAATGCCGAAAGAAATGCTTCCTAT

2 G L G T R F L P A T K A M P K E M L P I

3000 CGTTGATAAACCTACCATTCAATACATAATTGAAGAAGCTGTTGAAGCCGGTATTGAAGA

2 V D K P T I Q Y I I E E A V E A G I E D

3060 TATTATTATCGTAACAGGAAAAAGCAAGCGTGCGATTGAGGATCATTTTGATTACTCTCC

2 I I I V T G K S K R A I E D H F D Y S P

3120 TGAGCTTGAAAGAAACCTAGAAGAAAAAGGAAAAACTGAGCTGCTTGAAAAAGTGAAAAA

2 E L E R N L E E K G K T E L L E K V K K

3180 GGCTTCTAACCTGGCTGACATTCACTATATCCGCCAAAAAGAACCTAAAGGTCTCGGACA

2 A S N L A D I H Y I R Q K E P K G L G H

3240 TGCTGTCTGGTGCGCACGCAACTTTATCGGCGATGAGCCGTTTGCGGTACTGCTTGGTGA

2 A V W C A R N F I G D E P F A V L L G D

3300 CGATATTGTTCAGGCTGAAACTCCAGGGTTGCGCCAATTAATGGATGAATATGAAAAAAC

2 D I V Q A E T P G L R Q L M D E Y E K T

3360 ACTTTCTTCTATTATCGGTGTTCAGCAGGTGCCCGAAGAAGAAACACACCGCTACGGCAT

2 L S S I I G V Q Q V P E E E T H R Y G I

3420 TATTGACCCGCTGACAAGTGAAGGCCGCCGTTATCAGGTGAAAAACTTCGTTGAAAAACC

2 I D P L T S E G R R Y Q V K N F V E K P

3480 GCCTAAAGGCACAGCACCTTCTAATCTTGCCATCTTAGGCCGTTACGTATTCACGCCTGA

2 P K G T A P S N L A I L G R Y V F T P E

Bg1II

3540 GATCTTCATGTATTTAGAAGAGCAGCAGGTTGGCGCCGGCGGAGAAATTCAGCTCACAGA

2 I F M Y L E E Q Q V G A G G E I Q L T D

3600 CGCCATTCAAAAGCTGAATGAAATTCAAAGAGTGTTTGCTTACGATTTTGAAGGCAAGCG

2 A I Q K L N E I Q R V F A Y D F E G K R

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3660  TTATGATGTTGGTGAAAAGCTCGGCTTTATCACAACAACTCTTGAATTTGCGATGCAGGA
   2   Y  D  V  G  E  K  L  G  F  I  T  T  T  L  E  F  A  M  Q  D
3720  TAAAGAGCTTCGCGATCAGCTCGTTCCATTTATGGAAGGTTTACTAAACAAAGAAGAAAT
   2   K  E  L  R  D  Q  L  V  P  F  M  E  G  L  L  N  K  E  E  I
                                                NdeI
3780  CTAAGCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACATATGACGCATGTACG
   2                                             M  T  H  V  R
3840  CTTGACTACTCCAAAAGCGTTGACTTTCTTTCCAACGGAACATGAACTTACATACCTGCG
   2   L  T  T  P  K  A  L  T  F  F  P  T  E  H  E  L  T  Y  L  R
3900  GGACTTTGTAAAAACAGCACACCATAATATCCATGAGAAAACAGGCGCGGGCAGCGATTT
   2   D  F  V  K  T  A  H  H  N  I  H  E  K  T  G  A  G  S  D  F
                                          EcoRI
3960  TCTAGGCTGGGTGGACCTCCCTGAACATTATGATAAAGAAGAATTCGCGCGCATCCAAAA
   2   L  G  W  V  D  L  P  E  H  Y  D  K  E  E  F  A  R  I  Q  K
4020  AAGCGCGGAAAAAATCCAATCTGACTCTGATGTCTTGCTTGTTGTCGGCATCGGCGGTTC
   2   S  A  E  K  I  Q  S  D  S  D  V  L  L  V  V  G  I  G  G  S
4080  TTATCTTGGAGCGCGGGCAGCGATTGAAGCGCTGAATCACGCGTTTTATAACACTTTGCC
   2   Y  L  G  A  R  A  A  I  E  A  L  N  H  A  F  Y  N  T  L  P
4140  AAAAGCCAAACGCGGCAATCCGCAAGTCATTTTTAACTTCTCTATTAATGTGATTTCTAA
   2   K  A  K  R  G  N  P  Q  V  I  F  N  F  S  I  N  V  I  S  K
HindIII
4200  ATCAGGTACGACAACTGAACCTGCAATCGCTTTCCGTATTTTCCGCAAGCTTCTTGAAGA
   2   S  G  T  T  T  E  P  A  I  A  F  R  I  F  R  K  L  L  E  E
4260  GAAATACGGTAAAGAAGAAGCGAAAGCGCGGATTTATGCAACAACTGATAAAGAGCGCGG
   2   K  Y  G  K  E  E  A  K  A  R  I  Y  A  T  T  D  K  E  R  G
4320  CGCATTAAAAACGCTTTCTAACGAAGAAGGCTTTGAATCATTCGTAATTCCTGACGATGT
   2   A  L  K  T  L  S  N  E  E  G  F  E  S  F  V  I  P  D  D  V
4380  CGGCGGCCGTTATTCAGTTTTAACAGCTGTAGGTCTCTTGCCGATTGCTGTCAGCGGCGT
   2   G  G  R  Y  S  V  L  T  A  V  G  L  L  P  I  A  V  S  G  V
4440  CAACATTGACGACATGATGAAAGGCGCCCTGGATGCGAGCAAAGATTTTGCAACATCTGA
   2   N  I  D  D  M  M  K  G  A  L  D  A  S  K  D  F  A  T  S  E
4500  ACTGGAAGATAACCCAGCATACCAATATGCGGTTGTTCGCAATGTCCTTTATAATAAGGG
   2   L  E  D  N  P  A  Y  Q  Y  A  V  V  R  N  V  L  Y  N  K  G
4560  CAAAACAATTGAAATGCTCATCAACTACGAACCGGCGCTTCAATACTTTGCGGAATGGTG
   2   K  T  I  E  M  L  I  N  Y  E  P  A  L  Q  Y  F  A  E  W  W
4620  GAAGCAGCTGTTCGGAGAAAGCGAAGGGAAAGATGAGAAGGGCATTTATCCTTCTTCAGC
   2   K  Q  L  F  G  E  S  E  G  K  D  E  K  G  I  Y  P  S  S  A
4680  GAACTATTCAACAGACCTTCATTCTTTAGGCCAGTATGTACAAGAAGGCCGCAGAGATTT
   2   N  Y  S  T  D  L  H  S  L  G  Q  Y  V  Q  E  G  R  R  D  L
4740  ATTCGAAACGGTCCTGAACGTAGAGAAGCCTAAACATGAACTGACAATTGAGGAAGCGGA
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   2  F  E  T  V  L  N  V  E  K  P  K  H  E  L  T  I  E  E  A  D

4800  TAACGATCTTGACGGCTTGAACTATTTAGCCGGTAAAACTGTTGATTTCGTTAACAAAAA

   2  N  D  L  D  G  L  N  Y  L  A  G  K  T  V  D  F  V  N  K  K

4860  AGCATTCCAAGGTACAATGCTTGCCCATACAGACGGAAATGTTCCGAACTTAATCGTTAA

   2  A  F  Q  G  T  M  L  A  H  T  D  G  N  V  P  N  L  I  V  N

4920  CATTCCTGAGCTGAATGCATATACTTTTGGATACCTTGTATATTTCTTCGAAAAAGCCTG

   2  I  P  E  L  N  A  Y  T  F  G  Y  L  V  Y  F  F  E  K  A  C

4980  CGCGATGAGCGGTTACCTCCTTGGCGTCAATCCGTTTGACCAGCCTGGTGTAGAAGCGTA

   2  A  M  S  G  Y  L  L  G  V  N  P  F  D  Q  P  G  V  E  A  Y

5040  TAAAGTCAATATGTTTGCGTTACTCGGCAAACCTGGCTTTGAAGAGAAAAAAGCAGAGCT

   2  K  V  N  M  F  A  L  L  G  K  P  G  F  E  E  K  K  A  E  L

                                  NheI

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SphI         AgeI
                                                        (SEQ ID NO: 2)

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6000  CAGAAAAAAAGGATCTCAAGAAGATCCTTTGATCTTTTCTACGGGGTCTGACGCTCAGTG

6060  GAACGAAAACTCACGTTAAGGGATTTTGGTCATGAGATTATCAAAAAGGATCTTCACCTA

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                              PstI

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8640 GCCAAATTAAAATTTGGTTGTGTCGTAAATTCGATTGTGAATAGGATGTATTCACATTTC

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10020 GCAACTAAAATAAAAATGACGTTATTTCTATATGTATCAAGATAAGAAAGAACAAGTTCA

10080 AAACCATCAAAAAAAGACACCTTTTCAGGTGCTTTTTTTATTTTATAAACTCATTCCCTG

10140 ATCTCGACTTCGTTCTTTTTTTACCTCTCGGTTATGAGTTAGTTCAAATTCGTT
```

This plasmid has a molecular weight of 10194 bp and contains the various genes responsible for hyaluronic acid synthesis under the control of a strong T7 promoter of bacteriophage T7. The hasA sequence from *Streptococcus equi* is included between bases 196 and 1383, that of the tuaD gene between bases 1430 and 2873, the sequence coding for gtaB between bases 2905 and 3781, and that for gpi between bases 3824 and 5125.

Example 8

Restriction map of plasmid pT7hyal

When plasmid pT7hyal is cut with restriction enzymes it gives rise to a restriction map which corresponds to that expected after sequencing. In column 1 of FIG. **5**, it is shown that the cutting with enzyme EcoRI gives rise to three bands 4900 bp, 3240 bp and 2020 bp from plasmid; in column 2 it is shown that the cutting with EcoRI and HindIII gives rise to six bands 3290 bp, 2950 bp, 1660 bp, 1400 bp, 610 bp, and 290 bp; in column 3 it is shown that the cutting with HindIII gives rise to three bands 6240 bp, 2240 bp and 1690 bp, and in column 4 it is shown that, the cutting with restriction enzyme XbaI, single site gives rise to a single band (FIG. **5**).

Example 9

Check on Synthesis of Proteins which Lead to Hyaluronic Acid Synthesis

The two plasmids pPT7hasAtuaD and pPT7hasAtuaDgtaBpgi (pT7Hyal) were transformed into bacterial cells of *E. coli* BL21 DE3. After induction with IPTG, the cells were lysed, and the sample obtained was loaded into an SDS-PAGE to test for the presence of the proteins which lead to hyaluronic acid synthesis (FIG. **6**). The preparation in column 2 corresponds to cells which carried plasmid pPT7hasAtuaD: as shown in FIG. **6**, compared with the control colonies in column 1, column 2 presents a protein with a molecular weight of 54 kDa which corresponds to tuaD, and a protein with a weight of 42 kDa which corresponds to hasA. The samples in column 7 and 8 which carry plasmid pPT7hasAtuaDgtaBpgi produce, compared with control colonies 5 and 6, a protein with a molecular weight of 54 kDa which corresponds to tuaD, a protein with a molecular weight of 51 kDa which corresponds to pgi, a protein with a weight of 42 kDa which corresponds to hasA, and a protein with a molecular weight of 32 kDa which corresponds to gtaB. In conclusion, both plasmids produce the proteins of the expected molecular weight required for hyaluronic acid synthesis.

Example 10

Synthesis of Hyaluronic Acid in *E. coli* and Selection by IPTG Gradient

Plasmids pPT7 (control colony), pPT7hasAtuaD (colony 6) and pPT7hasAtuaDgtaBpgi (pT7Hyal—colony 2) were transformed into bacterial cells BL21 DE3. After 24 hours' growth at 37° C., the colonies were analysed for the production of hyaluronic acid. In solution, the cells which carry plasmids pPT7hasAtuaD (colony 6) or plasmids pPT7hasAtuaDgtaBpgi (colony 2) grow much more slowly, and after induction with IPTG only produce low levels of hyaluronic acid. The cells were then plated in the presence of IPTG (FIG. **7**).

The control colonies that carry plasmid pPT7 (and no hyaluronic acid synthesis gene) grow more easily, and are larger and flatter, than colony 6 and colony 2, in which the bacteria are engaged in producing hyaluronic acid; in fact, colonies 2 and 6 are shinier than the control as they produce hyaluronic acid. To select cells able to express high levels of hyaluronic acid, the cells were plated in the presence or absence of IPTG (FIG. **8**). In the presence of IPTG the majority of the colonies die, and only some survive, especially those close to the IPTG gradient formed. These cells were selected and replated in the presence of IPTG to establish their survival rate (FIG. **9**): all of them remained alive, maintaining their HA synthesis capacity.

The above statements are demonstrated by the fact that the cells of colonies 6 and 2 were cultured in solution for 48 hours in the presence of IPTG and 1% saccharose. 1 ml of this bacterial culture was centrifuged to obtain the precipitate, and the bacterial precipitate was then lysed in the presence of 0.1% SDS for 10 minutes. After adding 2 volumes of absolute ethyl alcohol, the result was as shown in FIG. **10**.

As will be seen, only colonies 6 and 2 give rise to a hyaluronic acid precipitate (which was tested with the carbazole test).

Example 11

Transformation of Plasmids pPT7hasAtuaD and pPT7hasAtuaDgtaBpgi into *Bacillus megaterium*

The *B. megaterium* used in the present invention is already pre-transformed with plasmid pT7-RNAP (QM B1551 MoBiTec) (this plasmid is able to replicate in both *E. coli* and *B. megaterium* because it contains two origins of replication which allow its propagation in both bacteria). It also contains resistance to ampicillin and chloramphenicol, which can be used for *E. coli* and *B. megaterium* respectively. The plasmid contains the sequence able to code for T7 RNA polymerase under the control of the inducible promoter for xylose, and also contains the repressor for the xylose promoter; if the cells are maintained in the absence of xylose, they are therefore unable to transcribe T7 RNA polymerase.

For the transformation of this bacterium it was necessary to remove its bacterial wall to obtain the protoplasts to use for the transformation. To remove the bacterial wall, 50 ml of LB medium were introduced into a 300 ml Erlenmeyer flask, and 1 ml of *Bacillus megaterium* grown overnight under aerobic conditions was added. When the cell density at OD578 reached the value of 1, the cells were centrifuged at 4500 rpm for 15 minutes. The cells were then suspended in 5 ml of 17.5 g/L of Antibiotic Medium no. 3, 500 mM saccharose, 20 mM sodium maleinate and 20 mM $MgCl_2$ pH6 (buffer SMMP). 50 ml of lysozyme 1 mg/ml in SMMP buffer were added and the mixture was maintained at 37° C. for 60 minutes, so as to remove the cell wall; the cells were then gently centrifuged at 1300 rpm for 10 minutes. The bacterial cells were then suspended in 5 ml of fresh SMMP buffer without stirring, as the protoplasts are sensitive to physical stress. This washing was repeated once more. After suspension, the protoplasts were ready to be used directly for the transformation or to be frozen at −80° C. in SMMP, which contains 15% glycerol. However, the transformations are much more efficient when the protoplasts are freshly prepared. For the transformation, 500 μl of protoplast suspension were mixed with 1 μg of plasmid DNA pPT7hasAtuaD or pPT7hasAtuaDgtaBpgi; 1.5 ml of PEG-P (40% w/v PEG6000 in 1×SMM) were then added, and the mixture was placed at ambient temperature for 2 minutes. 5 ml of SMMP were added, and the tubes were gently mixed by rotation.

The bacteria were centrifuged gently at 3000 rpm for 10 minutes at ambient temperature. The supernatant was discarded, and the almost invisible sediment contained the bacteria of interest. 500 μl of SMMP was added to the bacteria, which were then incubated for 90 minutes at 37° C. under slow stirring, at a maximum of 10 rpm; 2.5 ml of CR5 top agar were then prepared in sterile tubes in a hot bath at 43° C.

The CR5 top agar was prepared by mixing two components:

a) 51.5 g of saccharose, 3.25 g of MOPS and 0.33 g of NaOH in 250 ml of H_2O pH7.3, sterilised by filtration

b) 2.0 g of agar, 0.1 g of casaminoacids, 5 g of yeast extract and 142.5 ml of H_2O.

After autoclaving for 20 minutes, the two ingredients, cooled to 50° C., were mixed together.

After growth, 100 μl of the above disclosed cell preparation were added to 2.5 ml of top agar, mixed gently by rotating with both hands, and deposited on a pre-heated plate containing the antibiotic (4.5 μg/ml of chloramphenicol and 10 μg/ml of tetracycline). The mixture was incubated overnight at 37° C.; the colonies resulting larger or smaller, depending on their access to air.

Example 12

Expression of Hyaluronic Acid in *B. megaterium*

The transformed *B. megaterium* cells were cultured in LB medium with tetracycline and chloramphenicol up to an optical density at 578 nm of 0.4 at 37° C. The induction was conducted with the addition of 0.5% of D-xylose (w/v), followed by incubation at 37° C. The optical density of the bacteria was read every 30 minutes until the optical density at 600 nm reached 1.5; at this point the cells reached the steady state. These cells, as in the case of *E. coli*, are unable to produce hyaluronic acid directly after induction.

Example 13

System for the Selection of Hyaluronic Acid Secreting Cells

To obtain *B. megaterium* cells able to produce hyaluronic acid, the plate selection system presented for *E. coli* was employed, using xylose as inductor instead of IPTG. The cells which produce high levels of hyaluronic acid in the plate were then selected. Those cells survive, and can be

cultured. The supernatant contains the hyaluronic acid produced (its presence is confirmed by carbazole analysis when it is precipitated with two volumes of ethanol).

Example 14

Fermentation of transformed *B. megaterium* cells Selected on Gradient

Bacillus megaterium cells transformed with two genes pPT7hasAtuaD plasmid or with four genes pPT7hasAtuaDgtaBpgi plasmid, and selected on xylose gradient were cultured in a 20 1 fermenter in 5 1 of MM++ medium and glucose or saccharose as carbon source.

Xylose was added as inductor after the start of fermentation.

In the following some fermentation processes for the production of HA are illustrated, said processes mainly differing because of:

the starting source of carbon;

the added feed (glucose or saccharose);

the fermentation temperature (the temperature can be established in a range of from 20 to 38° C., preferably of from 25 to 35° C.);

time of fermentation.

Culture media used:

LB broth (Miller), pH 7

MM++ (Minimal Medium Bs), pH 7, containing per liter:

1 g $(NH_4)_2$ HPO_4; 1 g NH_4NO_3; 2.5 g K_2HPO_4; 2.5 g KH_2PO_4; 0.2 g $MgSO_47H_2O$; 0.01 g $FeSO_47H_2O$; 0.007 g $MnSO_47H_2O$.

Example 14a

Production of HA Having a Weight Average MW Comprised in the Range of 100-500 KD

The bacterial strain B. *Megaterium* (QM B1551), transfected with the plasmid pPT7hasAtuaDgtaBpgi selected on xylose gradient 0.5% w/v, as described in Example 13, was used.

Procedure: a single colony resistant to xylose was inoculated into 5 ml of sterile LB medium containing 5 mg/1 of tetracycline and the inductor. The culture was grown at 37° C., under stirring at 200 rpm.

After 8 hours, 50 μl of this culture were inoculated into a flask containing 50 ml of the medium mentioned above (containing the inductor), and it was made to grow under the same conditions described above.

Subsequently, spent further 14-16 hours, 2 ml of this culture were inoculated into a flask containing 500 ml of the medium above, and it was made to grow under the same conditions until reaching a $D.O.^{600nm}$ of 0.6-0.8.

500 ml of the culture thus obtained were then inoculated in the fermenter containing MM++ medium, and the fermentation conditions involved maintaining the culture under stirring at 600 rpm, aeration with 20-24 litres of air/min, a temperature of 37° C. (the temperature of fermentation can be established in a range between 25° C. and 38° C.), and a pH of 6.9 to 7.1. The initial source of carbon was 2% saccharose.

After 4 hours of fermentation, a 2% saccharose supply was added. At 24 hours of fermentation, xylose was added to a final concentration of 0.5%; this induction proceeded for 4 hours; at the end, 10% saccharose was added in steps.

At the end of fermentation (130 hours), the bacterial culture was discharged and centrifuged at 7500 rpm at 8° C. for 20 minutes.

The fermentation broth thus obtained, clarified as free of the cellular component, was analyzed to determine the concentration of HA with the carbazole method (Bitter and Muir, 1962, *Anal. Biochem.* 4:330-334).

Results: The analysis resulted in a concentration of HA of 3.5 g/l.

Determination of weight average molecular weight MW:

For its analysis it was used the method of the intrinsic viscosity (as described in Terbojevich et al., *Carbohydr. Res.* 1986, 363-377, incorporated herein by reference).

Results: the analyzed HA sample showed a weight average molecular weight MW in the range of 100-300 KD.

Example 14b

Production of HA Having a Weight Average MW Comprised in the Range of $1\times10^{6-}2\times10^{6}$ D

The bacterial strain B. *Megaterium* (QM B1551), transfected with the two genes plasmid pPT7hasAtuaD and with the four genes plasmid pPT7hasAtuaDgtaBpgi, selected on xylose gradient, as described in Example 13, was used.

Procedure: for each plasmid which was used, a single colony resistant to xylose was processed as indicated in example 14a. The initial source of carbon was saccharose at 2%: in this example the further supply was glucose (further experimental tests showed that it can be substituted with equal or lower amounts of saccharose). The fermentation conditions were the same as those used in example 14a with the only difference of the fermentation temperature: 25° C.

The culture media used for the fermentation were those disclosed according to example 14a.

At the end of the process (ended after 24 hours), the fermentation broth was analyzed to determine the concentration of HA with the carbazole method.

Results: *B. Megaterium* (QM B1551), transfected with the two genes plasmid pPT7hasAtuaD: the analysis resulted in a concentration of HA of 2.5 g/1;

B. Megaterium (QM B1551), transfected with the four genes plasmid pPT7hasAtuaDgtaBpgi: the analysis resulted in a concentration of HA of 3.2 g/1;

Determination of weight average molecular weight MW:

For its analysis it was used the method of the intrinsic viscosity as indicated in the previous example 14a.

Results: the analyzed HA sample produced by *B. Megaterium* transfected with the two genes plasmid showed a weight average molecular weight MW in the range of $1.3\times10^{6}-1.7\times10^{6}$D;

the analyzed HA sample produced by *B. Megaterium* transfected with the four genes plasmid showed a weight average molecular weight MW in the range of $1.6\times10^{6}-2\times10^{6}$D.

The system engineered in *B. megaterium* is inducible, so the fermentation process can be continued by stimulating the production of HA to obtain the desired weight average molecular weight MW; fermentation times between 80 and 160 hours result in a medium-low weight average molecular weight MW, comprised in the range between 100-500 KD, fermentation times between 40 and 80 hours result in a weight average molecular weight in the range between 500-1000 KD, fermentation times between 12 and 40 hours result in a weight average molecular weight MW in the range $1\times10^{6}-3\times10^{6}$ D.

With the experiments and the results obtained above, the Applicant has demonstrated to have perfected a system of production of HA in *B. megaterium* by plasmid vectors by:

- engineering of 2 genes (or 4 genes) plasmid vectors for the synthesis of enzymes needed for the production of said polysaccharide, whose gene control is placed under the control of strong promoter T7 of bacteriophage T7;
- perfecting a system of selection of these transfected strains for the production of stable, viable, replicating and HA secreting strains;
- creating an inducible system of HA production, thus controllable both in order to obtain high concentrations of HA and for the production of said polysaccharide at different weight average molecular weight MW.

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The invention claimed is:

1. A process for the preparation of hyaluronic acid in *Bacillus megaterium*, comprising the following steps:

(a) culturing bacterial host cells of *Bacillus megaterium*, transformed in a stable way with the T7 RNA polymerase system under conditions suitable for the production of hyaluronic acid in the presence of xylose as an inductor, wherein said bacterial host cells are characterised by being further transformed with:

(i) at least one episomal plasmid vector comprising a sequence coding for the enzyme hyaluronan synthase and a sequence coding for the enzyme UDP-glucose dehydrogenase in tandem under the control of the strong inducible T7 promoter; or

(ii) at least one episomal plasmid vector comprising a sequence coding for the enzyme hyaluronate synthase, a sequence coding for the enzyme UDP-glucose dehydrogenase, a sequence coding for the enzyme UDP-glucose pyrophosphorylase and a sequence coding for the enzyme glucose 6 phosphate isomerase, under the control of the strong inducible T7 promoter;

(b) recovering hyaluronic acid from the culture medium,

wherein such bacterial host cells of *Bacillus megaterium* transformed in a stable way with the T7 RNA polymerase system and with plasmid vector (i) or (ii) able to produce hyaluronic acid of step a) are pre-selected on a xylose gradient.

2. The process according to claim **1**, wherein the xylose inducer is added to a concentration of between 0.1% and 10% w/v.

3. The process of claim **2**, in which the xylose inducer is added to a concentration of between 0.5% and 1% w/v.

4. The process according to **1**, wherein said bacterial host cells of *Bacillus megaterium* transformed with the T7 RNA polymerase system belong to *B. megaterium* strain QM B1551 or DSM319.

5. The process according to claim **1**, wherein the sequence coding for the enzyme hyaluronan synthase (hasA) is obtained from a strain of *Streptococcus*, and the sequences coding for enzymes UDP-glucose dehydrogenase(hasB or tuaD), UDP-glucose pyrophosphorylase (gtaB) and glucose 6 phosphate isomerase (pgi or hasE) are obtained from *Bacillus subtilis*.

6. The process according to claim **1**, in which the sequences coding for the enzyme UDP-glucose dehydrogenase, hyaluronan synthase, UDP-glucose pyrophosphorylase and glucose 6 phosphate isomerase are operatively linked to an upstream Shine-Dalgarno sequence.

7. The process according to claim **1**, wherein said plasmid vector (i) comprises the nucleotide sequence of SEQ ID NO:1.

8. The process according to claim **1**, wherein said plasmid vector (ii) comprises the nucleotide sequence of SEQ ID NO:2.

9. The process according to claim 1, wherein the fermentation time is in the range between 80 and 160 hours and the product HA has a weight average molecular weight in the range 100-500 KDa.

10. The process according to claim 1, wherein the fermentation time is in the range between 40 and 80 hours and the product HA has a weight average molecular weight in the range 500-1000 KDa.

11. The process according to claim 1, wherein the fermentation time is in the range between 12 and 40 hours and the product HA has a weight average molecular weight in the range 1×10^6–3×10^6 D.

12. A process for the preparation of hyaluronic acid in *Escherichia coli*, comprising the following steps:

(a) culturing bacterial host cells of *Escherichia coli*, transformed in a stable way with the T7 RNA polymerase system under conditions suitable for the production of hyaluronic acid in the presence of isopropyl-β-thio-galactopyranoside (IPTG) as an inductor, wherein said bacterial host cells are characterised by being further transformed with:

(i) at least one episomal plasmid vector comprising a sequence coding for the enzyme hyaluronan synthase and a sequence coding for the enzyme UDP-glucose dehydrogenase in tandem under the control of the strong inducible T7 promoter; or

(ii) at least one episomal plasmid vector comprising a sequence coding for the enzyme hyaluronate synthase, a sequence coding for the enzyme UDP-glucose dehydrogenase, a sequence coding for the enzyme UDP-glucose pyrophosphorylase and a sequence coding for the enzyme glucose 6 phosphate isomerase, under the control of the strong inducible T7 promoter;

(b) recovering hyaluronic acid from the culture medium, wherein such bacterial host cells of *Escherichia coli* transformed in a stable way with the T7 RNA polymerase system and with plasmid vector (i) or (ii) able to produce hyaluronic acid of step a) are pre-selected on an IPTG gradient.

13. The process according to claim 12, wherein the IPTG inducer is added to a concentration of between 0.1 mM and 10 mM.

14. The process of claim 13, in which the IPTG inducer is added to a concentration of between 0.4 mM to 1.0 mM.

15. The process according to claim 12, wherein said bacterial host cells of *Escherichia coli* transformed with the T7 RNA polymerase system belong to *E. coli* strain BL21 DE3.

16. The process according to claim 12, wherein the sequence coding for the enzyme hyaluronan synthase (hasA) is obtained from a strain of *Streptococcus*, and the sequences coding for enzymes UDP-glucose dehydrogenase(hasB or tuaD), UDP-glucose pyrophosphorylase (gtaB) and glucose 6 phosphate isomerase (pgi or hasE) are obtained from *Bacillus subtilis.*

17. The process according to claim 12, in which the sequences coding for the enzyme UDP-glucose dehydrogenase, hyaluronan synthase, UDP-glucose pyrophosphorylase and glucose 6 phosphate isomerase are operatively linked to an upstream Shine-Dalgarno sequence.

18. The process according to claim 12, wherein said plasmid vector (i) comprises the nucleotide sequence of SEQ ID NO:1.

19. The process according to claim 12, wherein said plasmid vector (ii) comprises the nucleotide sequence of SEQ ID NO:2.

20. A plasmid vector comprising a strong inducible bacteriophage T7 promoter operationally linked to a sequence coding for a hyaluronan synthase enzyme and a sequence coding for a UDP-glucose dehydrogenase enzyme in tandem.

21. The plasmid vector according to claim 20, wherein said sequence coding for the enzyme hyaluronan synthase is a hasA gene from *Streptococcus zooepidemicus*, and said sequence coding for the enzyme UDP-glucose dehydrogenase is a tuaD gene from *Bacillus subtilis.*

22. The plasmid vector according to claim 21, comprising the nucleotide sequence SEQ ID NO:1.

23. A plasmid vector comprising a strong inducible bacteriophage T7 promoter operationally linked to a sequence coding for a hyaluronate synthase enzyme, a sequence coding for a UDP-glucose dehydrogenase enzyme, a sequence coding for a UDP-glucose pyrophosphorylase enzyme and a sequence coding for a glucose 6 phosphate isomerase enzyme.

24. The plasmid vector according to claim 23, wherein said sequence coding for the enzyme hyaluronan synthase is a hasA gene from *Streptococcus zooepidemicus*, said sequence coding for the enzyme UDP-glucose dehydrogenase is a tuaD gene from *Bacillus subtilis*, said sequence coding for the enzyme UDP-glucose pyrophosphorylase is a gtaB gene from *Bacillus subtilis* and said sequence coding for the enzyme glucose 6 phosphate isomerase is a pgi gene from *Bacillus subtilis.*

25. The plasmid vector according to claim 24, comprising the nucleotide sequence SEQ ID NO:2.

26. The plasmid vector according to claim 23, wherein the sequence coding for the enzyme UDP-glucose dehydrogenase, hyaluronan synthase, UDP-glucose pyrophosphorylase and glucose 6 phosphate isomerase are operatively linked to an upstream Shine-Dalgarno sequence.

27. A recombinant host bacterial cell belonging to the genus *Bacillus* previously transformed with the T7 RNA polymerase system, comprising at least one plasmid vector according to claim 20.

28. The recombinant host bacterial cell according to claim 27, which is *Bacillus megaterium.*

29. A method for obtaining recombinant host bacterial cells, according to claim 27, which are capable of producing high levels of hyaluronic acid, comprising selecting bacterial host cells transformed with a plasmid vector comprising a strong inducible bacteriophage T7 promoter operationally linked to a sequence coding for a hyaluronan synthase enzyme and a sequence coding for a UDP-glucose dehydrogenase enzyme in tandem and transformed with a T7 RNA polymerase system, on a xylose gradient.

30. A recombinant host bacterial cell belonging to the genus *Escherichia* previously transformed with the T7 RNA polymerase system, comprising at least one plasmid vector according to claim 20.

31. The recombinant host bacterial cell according to claim 30, which is *Escherichia coli.*

32. A method for obtaining recombinant host bacterial cells, according to claim 30, which are capable of producing high levels of hyaluronic acid, comprising selecting bacterial host cells transformed with a plasmid vector comprising a strong inducible bacteriophage T7 promoter operationally linked to a sequence coding for a hyaluronan synthase enzyme and a sequence coding for a UDP-glucose dehydrogenase enzyme in tandem and transformed with a T7 RNA polymerase system, on an IPTG gradient.

* * * * *

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 9,695,453 B2
APPLICATION NO. : 14/884274
DATED : July 4, 2017
INVENTOR(S) : Vincenza Corsa et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

On the Title Page

At item (72), Inventors, change "**Vincenza Corsa**, Albano Terme (IT)" to --**Vincenza Corsa**, Abano Terme (IT)--.

At item (63), **Related U.S. Application Data**, change "Continuation of application No. 13/821,953, filed as application No. PCT/EP2011/065641 on Apr. 15, 2013, now Pat. No. 9,163,270." to --Continuation of application No. 13/821,953, filed as application No. PCT/EP2011/065641 on Sep. 9, 2011, new Pat No. 9,163,270.--.

Signed and Sealed this
Twenty-fourth Day of October, 2017

Joseph Matal

Joseph Matal
Performing the Functions and Duties of the
Under Secretary of Commerce for Intellectual Property and
Director of the United States Patent and Trademark Office